STUDIES ON THE REGULATION OF CONIDIATION IN SPECIES OF *Trichoderma*

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A characteristic feature of species of *Trichoderma* is the production of concentric rings of conidia in response to alternating light-dark conditions. In response to a single burst of light, a single ring of conidia forms at what was the colony perimeter. On the basis of these observations, competency to photoconidiate has been proposed to be due to the age and metabolic rate of the hyphal cell. In this study, conidiation was investigated in five biocontrol isolates (*T. hamatum*, *T. atroviride*, *T. asperellum*, *T. virens* and *T. harzianum*) using both a morphological and molecular approach. All five isolates produced concentric conidial rings under alternating light-dark conditions on potato-dextrose agar (PDA), however, in response to a 15 min burst of blue light, only *T. asperellum* and *T. virens* produced a clearly, defined conidial ring which correlated with the colony margin at the time of light exposure. Both *T. harzianum* and *T. hamatum* photoconidiated in a disk-like fashion and *T. atroviride* produced a broken ring with a partially filled in appearance. On the basis of these results, it was postulated that competency to photoconidiate is a factor of the metabolic state of the hyphal cell rather than chronological age or metabolic rate.

The influence of the source of nitrogen on photoconidiation was assessed on pH-buffered (pH 5.4) minimal medium (MM) amended with glutamine, urea or KNO_3. In the presence of glutamine or urea, *T. asperellum* and *T. harzianum* conidiated in a disk, whereas, when KNO_3 was the sole nitrogen source, a ring of conidia was produced. Further, in the presence of increasing amounts of glutamine, the clearly defined photoconidial ring produced on PDA by *T. asperellum* became disk-like. These results clearly demonstrated that primary nitrogen promotes photoconidiation in these isolates and strongly suggests that competency of a hyphal cell to conidiate in response to light is dependent on the nitrogen catabolite repression state of the cell.

The experiments were repeated for all five isolates on unbuffered MM. Differences were apparent between the buffered and unbuffered experiments for *T. atroviride*. No photoconidiation was observed in *T. atroviride* on buffered medium whereas on unbuffered medium, rings of conidia were produced on both primary and

secondary nitrogen. These results show that photoconidiation in *T. atroviride* is influenced by the buffering capacity of the medium.

Conidiation in response to light by *T. hamatum* and *T. virens* was absent in all nitrogen experiments, regardless of the nitrogen source and buffering capacity, whereas both isolates conidiated in response to light on PDA. These results imply that either both sources of nitrogen are required for photoconidiation, or a factor essential for conidiation in these two isolates was absent in the minimal medium.

Mycelial injury was also investigated in five biocontrol isolates of *Trichoderma*. On PDA, all isolates except *T. hamatum* conidiated in response to injury. On nitrogen amended MM, conidiation in response to injury was again observed in all isolates except for *T. hamatum.* In *T. atroviride*, injury-induced conidiation was observed on all medium combinations except the pH-buffered MM amended with glutamine or urea and *T. virens* conidiated in response to injury on primary nitrogen only, regardless of the buffering capacity. These results have revealed conidiation in response to injury to be differentially regulated between isolates/species of *Trichoderma*.

On unbuffered MM amended with glutamine or urea, conidiation in response to injury occurred at the colony perimeter only in *T. atroviride*. It was hypothesised that the restriction of conidiation to the perimeter may be due to changes in the pH of the agar. The experiment was repeated and the pH values of the agar under the growing colony measured at the time of light induction (48 h) or injury (72 h). The areas under the hyphal fronts were acidified to below the starting value of the medium (pH 5.4) and the centres of the plates were alkalinised. The region of acidification at the time of stimuli correlated with the production of conidia, which implicates a role for cross-regulation of conidiation by the ambient pH.

The influence of the ambient pH on injury-induced conidiation was investigated in *T. hamatum* and *T. atroviride* on MM amended with glutamine and PDA, pH-buffered from pH 2.8 to 5.6. Thickening of the hyphae around the injury site was observed at the lowest pH values on MM in both *T. atroviride* and *T. hamatum*, however no conidia were produced, whereas both *Trichoderma* species conidiated on pH-buffered PDA in a strictly low pH-dependent fashion. This is the first observation of injury-induced conidiation in *T. hamatum*.

The influence of the ambient pH on photoconidiation was assessed in *T. hamatum*, *T. atroviride* and *T. harzianum* using both buffered and unbuffered PDA from pH 2.8 to 5.2. On buffered PDA, no conidiation in response to light was observed above pH 3.2 in *T. hamatum*, above 4.0 in *T. atroviride* and above 4.4 in *T. harzianum*, whereas on unbuffered PDA it occurred at all pH values tested. It was postulated that conidiation at pH values above 4.4 on unbuffered PDA was due to acidification of the agar. The pH values of the agar under the growing colony were measured at the time of light exposure and in contrast to the MM with glutamine experiments, alkalisation of the agar had occurred in both *T. atroviride* and *T. hamatum*. No change in medium pH was recorded under the growing *T. harzianum* colony. These results indicate that low pH-dependence of photoconidiation is directly related to the buffering capacity of the medium.

Recent studies have linked regulation of conidiation in *T. harzianum* to Pac1, the PacC orthologue. In fungi, PacC regulates gene expression in response to the ambient pH. In these studies pH-dependent photoconidiation occurred only on buffered PDA and on unbuffered PDA conidiation occurred at significantly higher ambient pH levels. It is proposed that the influence of ambient pH on conidiation in the isolates used in this study is not due to direct Pac1 regulation.

The *T. harzianum* isolate used in this study produced profuse amounts of the yellow anthraquinone pachybasin. Production of this secondary metabolite was strictly pH-dependent, irrespective of the buffering capacity of the medium. Studies in *T. harzianum* have linked Pac1 regulation to production of an antifungal α-pyrone. pH-dependence on both buffered and unbuffered media strongly suggests that pachybasin production may also be under the control of Pac1.

Photoconidiation studies on broth-soaked filter paper, revealed rhythmic conidiation in the pachybasin producing *T. harzianum* isolate. Diffuse rings of conidia were produced in dark-grown cultures and, in cultures exposed to light for 15 min at 48 h, the rings were clearly defined. These results show that conidiation is under the control of an endogenous rhythm in *T. harzianum* and represent the first report of circadian conidiation in a wild-type *Trichoderma*.

A Free-Running Rhythm (FRR) assay was used to investigate rhythmic gene expression in *T. atroviride* IMI206040 and a mutant derivative, in which the *wc-2* orthologue, *blr-2*, was disrupted. Over a 3 d period, expression of *gpd*, which encodes

the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, oscillated with a period of about 48 h. In the Δ*blr-2* mutant, the *gpd* rhythm was absent. These results revealed that in *T. atroviride*, *gpd* expression is under the control of an endogenous clock and that clock-regulated expression of *gpd* is associated with a functional BLR complex.

Using degenerate primers, a portion of *frq*, which encodes the *N. crassa* clock oscillator FREQUENCY, was isolated from *T. atroviride* and used to probe the FRR assay northern blots. No *frq* expression was detected at any time point, which suggests that the circadian clock in *Trichoderma* does not involve FREQUENCY.

In a concurrent study, orthologues of *rco-1* (*rcoT*) were isolated and sequenced from *T. atroviride* and *T. hamatum* using a combination of degenerate, inverse and specific PCR. RcoT is an orthologue of the yeast global co-repressor Tup1 and in the filamentous fungi, RcoT orthologues have been demonstrated to negatively regulate conidiation. Genomic analysis of all available *rcoT* orthologues revealed the conservation of *erg3*, a major ergosterol biosynthesis gene, upstream from *rcoT* in ascomycetous filamentous fungi, but not in the ascomycetous yeast or in the basidiomycetes.

These studies have significantly contributed to our understanding of the regulatory factors controlling conidiation in *Trichoderma* and have multiple implications for *Trichoderma* biocontrol; most notable the promotion of conidiation by primary nitrogen and low pH. Incubation conditions can be altered to suit the nitrogen and pH preferences of a biocontrol strain in order to promote cost effective conidial production, however this is not easily achieved in the soil, where the biocontrol strain must perform in a highly buffered environment optimised for plant growth. Successful use of *Trichoderma* biocontrol strains may involve the screening and targeting of strains to the appropriate pH conditions or the selection of new strains on the basis of capacity to perform under a given range of conditions.

Keywords: *Trichoderma*, conidiation, nitrogen catabolite repression, pH regulation, anthraquinone, pachybasin, circadian, free-running rhythm, *blr-1*, *frq*, *gpd*, *rcoT*, *erg3*, *con-10*

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To my mother, Johanna Antonia,
and my father, Emilius Johannes,
Thank you for giving me life
and the strength to live it

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Abbreviations

A	adenine
ascn#	accession number
bp	base pairs
C	cytosine
cm	centimetres
d	days
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
dGTP	deoxyguanosine triphosphate
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetra-acetate
ETS	external transcribed spacer
G	guanine
g	gram
gDNA	genomic deoxyribonucleic acid
h	hours
ITS	internal transcribed spacer
kb	kilobase
kDa	kilodalton
L	litre
M	molar
min	minutes
mg	milligram
mol	mole
mL	millilitre
mm	millimetre
mM	millimolar
MM	minimal media
mRNA	messenger ribonucleic acid

N	any nucleotide (A, C, G or T)
ng	nanogram
nm	nanometre
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
pers. comm.	personal communication
pg	picogram
pmol	picomole
psi	pounds per square inch
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
s	seconds
SDS	sodium dodecyl sulphate
SSC	sodium chloride sodium citrate
T	thymine
Tris	Tris(hydroxymethyl) aminomethane
TSP	transcription start point
U	units
UP-PCR	universally primed polymerase chain reaction
UV	ultraviolet
V	volts
x*g*	relative centrifugal force
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactosidase
°C	degrees celsius
α	alpha
β	beta
μg	microgram
μL	microlitre
μM	micromolar
μmol	micromoles

Prologue

Two approaches were taken to explore regulation of conidiation in Trichoderma. In the first approach, a morphological analysis of conidiation was undertaken, in which the effect of various abiotic factors were investigated. The initial experiments yielded some unexpected results, which were explored in a further series of experiments, including an analysis of circadian rhythms. These studies comprise Chapters 2 through 3, and include a separate literature review (Chapter 1).

In concurrent work, a molecular study was undertaken to isolate and characterise a putative regulator of conidiation (*rcoT*, orthologues of *rco-1*). One of the objectives of this work was to develop a series of *rcoT* loss of function mutants and to evaluate them in the context of the discoveries and conclusions from Chapters 2 through 3. Unfortunately, verifiable mutants were not obtained; weakening the linkage between the two sections of work. Consequently, Chapter 4, is included as a separate part of the thesis and, accordingly, it contains its own literature review.

Chapter One
Introduction

1.1 Reproduction in Fungi

Fungi reproduce both sexually and asexually producing a vast array of structures which have evolved over time to suit habitat and in some cases host. These structures are of great economic and health importance to society. Approximately 48% of the world's food crop yield is lost due to plant diseases, of which the majority are caused by fungi (Agrios, 2005). For most fungal diseases, the primary sources of inoculum are sexual and/or asexual spores. Once infection is established, sporulation often continues providing secondary inoculum which, if unchecked, can worsen the crop loss dramatically. As well as economic losses, fungi can have positive economic benefits for agriculture, such as biocontrol of plant diseases. Numerous fungi have been successfully developed as biocontrol agents of plant diseases and the majority of these are sold as spore preparations (Chernin & Chet, 2002). Many fungi causing human diseases undergo reproduction inside the host, and the reproductive structures often represent highly infectious propagules (Nielson & Heitman, 2007). The importance of fungal reproduction in a multitude of disciplines has stimulated considerable research into the physiology, biochemistry and genetics underlying reproduction in both yeast and the filamentous fungi. Some processes are well understood, and others are only now able to be explored with the advent of molecular cloning. What is beginning to emerge is commonality between species as well as vast differences. The more species we investigate, the more commonalities will be identified, and through this we should be able to understand the fundamental processes involved in reproductive morphogenesis in fungi.

1.2 The Genus *Trichoderma*

Trichoderma spp. are ubiquitous soil fungi found in all climatic zones (Papavizas, 1985; Klein & Eveleigh, 1998). They are common in agricultural, grassland, forest, saline and desert soils, and are particularly prevalent in the humic layer of hardwood forests where they represent up to 3% of all fungal propagules. *Trichoderma* spp. are described as anamorphs of the ascomycetous order Hypocreales (Samuels, 1996). The majority of

proven teleomorphs are of the genus *Hypocrea*, however two other Hypocreales genera (*Podostroma* and *Sarawakus*) have *Trichoderma* anamorphs.

The taxonomy of *Trichoderma* is confusing, complex and incomplete (Samuels, 1996; Gams and Bissett, 1998; Kindermann *et al.*, 1998). Species identification has traditionally been based on morphological and cultural characteristics, and while these are still the primary means of identification, many taxonomists considered them inadequate for correct identification (Samuels, 1996; Kindermann *et al.*, 1998). The literature contains multiple examples of mis-identification, including some of the most well characterised isolates (Kullnig *et al.*, 2001). The first attempt at a morphological taxonomy was by Rifai (1969), who described *Trichoderma* spp. in terms of nine aggregate species. A more detailed study by Bissett (1991) identified species in terms of five sections, and also included some species originally considered to be *Gliocladium*. With the advent of molecular taxonomic techniques, the genus has again been redefined, some morphological sections merged, and even a suggestion made to replace morphological sections with phylogenetic groupings (Kindermann *et al.*, 1998). Various morphological characters are used to differentiate *Trichoderma* spp. into sections and species, however these descriptors are not absolute, and correct identification should be by a combination of morphological and genetic characters. In general, *Trichoderma* spp. in culture exhibit rapid growth and produce abundant powdery, green enteroblastic conidia from phialides (Figure 1.1) (Samuels, 1996; Gams & Bissett, 1998).

Trichoderma spp. are considered superior saprophytes able to enzymatically attack and metabolise a wide range of substrates, which has led to their exploitation in industry and agriculture. *Trichoderma* spp. produce cellulases for the metabolism of cellulosic substances and are able to attack and metabolise other fungi as a source of food (Samuels, 1996). Their antagonistic abilities towards pathogenic fungi have been harnessed globally as a means of biocontrol of soil-borne fungal phytopathogens and, as a consequence, *Trichoderma* spp. represent one third of all commercial fungal biocontrol agents. Commercial preparations of *Trichoderma* spp. for biocontrol consist of bulk produced conidia, whereas good biocontrol activity in the environment relies upon the fungus remaining vegetative and thus antagonistically active. Therefore, efficient and effective use of *Trichoderma* biological controls agents (BCAs) involves achieving a balance between ample cost-effective conidiation during production and vigorous vegetative growth during usage.

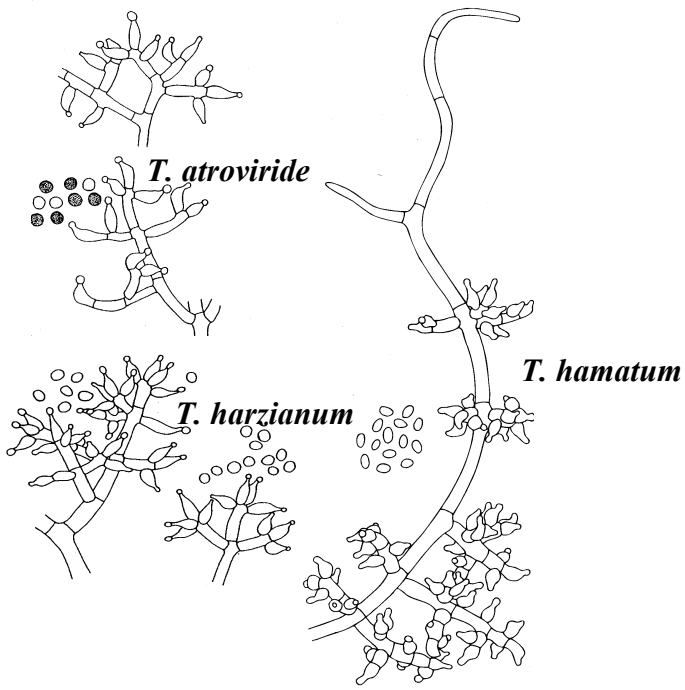


Figure 1.1. *Trichoderma* spp. hyphal and conidiophore morphology, from Gams and Bissett (1998).

1.3 Conidial Morphogenesis in *Trichoderma*

Conidiation in *Trichoderma* can be stimulated *in vitro* through the manipulation of nutrients, exposure to light and by injury to the mycelium (Betina & Farkaš, 1998; Casas-Flores *et al.*, 2004). The use of light to stimulate conidiation has allowed for precise studies of the stages of conidial morphogenesis, revealing a highly ordered progression. Conidiogenesis proceeds in a series of five stages, which has been described in *T. viride*, *T. harzianum* and *T. atroviride* (Figure 1.2) (Gressel & Galun, 1967; Betina & Zajacová, 1978a, b; Puyesky *et al.*, 1999).

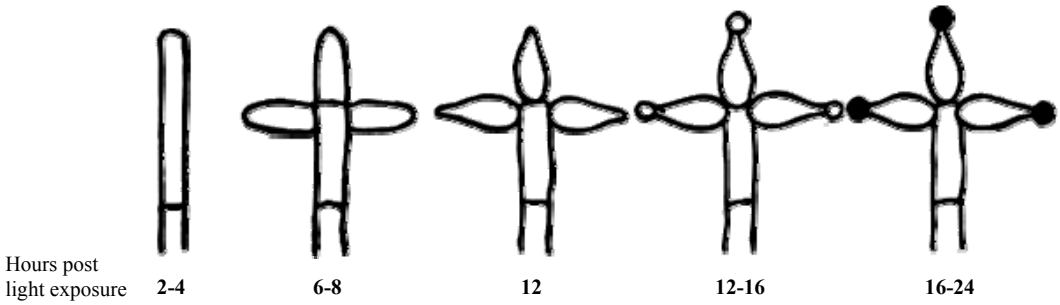


Figure 1.2. The five stages of conidiogenesis in *Trichoderma* spp., adapted from Betina & Zajacová (1978b). 2-4 h: a dense ring of aerial hyphae appears. 6-8 h: vertical mycelium branches. 12 h: formation of phialides. 12-16 h: hyaline spores appear. 16-24 h: intensive conidiation and pigmentation.

1.4 Inducers of Conidiation

1.4.1 Abiotic Influences

Conidia of *Trichoderma* spp. are produced commercially under cultural conditions in which the nutritional status and pH of the medium and the incubation temperature have been optimised for each isolate. In general, light is not utilised as an inducer, with induction brought about by nutritional rather than photo-influences. This has led to much research on the nutritional requirements of *Trichoderma* isolates for optimal conidiation and growth. Though many isolates have been investigated, no single set of optimal parameters has been able to be assigned to a given species. It would appear, therefore, that optimal cultural conditions for stimulating conidiation are isolate-specific.

Carbon and nitrogen status and pH of the medium have been demonstrated to be the main cultural parameters having an influence on conidiation other than light. Conditions which favoured growth generally decreased conidiation (Brian and Hemming, 1950; Jackson *et al.*, 1991). Lewis and Papavizas (1983) and Monga (2001) demonstrated that conidiation in liquid culture was favoured by primary carbon and organic nitrogen sources, whereas secondary carbon and inorganic nitrogen sources favoured mycelial growth.

The relative ratio between carbon and nitrogen has been shown to be highly influential on conidiation and growth. Aube and Gagnon (1969) observed that conidiation decreased with increasing nitrogen levels and Jackson *et al.* (1991) found that mycelial biomass increased significantly as the nitrogen levels decreased. This concept was investigated further by Gao *et al.* (2007) who demonstrated an interactive effect on conidiation in *T. viride* between the level of carbon and the C:N ratio. Alone or in combination with different amounts of nitrogen, 6 g/L carbon (sucrose) produced the optimum amount of conidia. The rate of conidiation did not vary significantly as the C:N (soy peptone) ratio rose from 10:1 to 80:1, however at 160:1 the rate of conidiation more than doubled compared to previous values. At 6 and 8 g/L carbon, mycelial growth was highest at a C:N ratio of 10:1. Whereas, at 12 g/L carbon, the optimum C:N ratio rose to 40:1. These results showed that higher amounts of nitrogen favoured mycelial growth, whereas nitrogen limitation favoured conidiation.

The initial pH of the medium has also been demonstrated to have an effect on conidiation and, unlike the C:N ratio, pH levels which favoured conidiation were shown to also favour mycelial growth (Brian & Hemming, 1950; Aube & Gagnon, 1969; Lewis & Papavizas, 1983; Bastos, 2001). The pH optimum varied between isolates from pH 5.5 to 6.8. While the initial pH of the medium had some influence on conidiation and growth, the *Trichoderma* culture itself altered the pH of the medium (Lewis and Papavizas, 1983). Changes in the medium pH were dependent on the carbon and nitrogen sources; with primary carbon and organic nitrogen the pH decreased, whereas alkalisation was observed in the secondary carbon and inorganic nitrogen experiments.

1.4.2 Light-induced Conidiation

1.4.2.1 Light-stimulated Conidiation Rings

Conidiation in *Trichoderma* can also be stimulated by light. Though this mechanism of induction is not wholly exploited in commercial preparations of conidia, it does provide a means for conducting controlled, precise experiments on conidiation. In continuous light, conidiation occurs across the colony, whereas under alternating light-dark conditions, concentric rings of conidial formation can be seen in cultures. Gutter (1957) put forward the theory of competency as an explanation for the production of conidiation rings. Competency is where only hyphal cells of a specific age are responsive to light. This was first described in an early study on *T. viride* conidiation, which used potato-dextrose agar (PDA) as the medium and spore suspensions as the inoculum (Gutter, 1957). Position of the induced conidiophore ring relative to the colony edge at the time of induction indicated a minimum hyphal age of approximately 10 h was necessary for induction in plate cultures of four *T. viride* isolates. Dark-grown mycelium older than 20 h was no longer responsive to the light stimulus. However, one culture (isolate 5) had little or no competency threshold and, unlike the other isolates, no dark grown mycelium was able to be induced, which suggested a light requirement for production of components necessary for photoconidiation in this isolate.

Studies on nucleic acid synthesis during photoinduction have linked hyphal cell competency to the metabolic rate of the cell rather than hyphal age *per se*. Gressel and Galun (1967) reported a 16 h minimum before *T. viride* cultures were able to conidiate in response to light and a lag phase of about 24 h before subsequent light doses were effective. In contrast to Gutter (1957), Gressel and Galun (1967) used filter paper soaked in potato-dextrose broth (PDB) as the medium and mycelial plugs as the

inoculum. Using labelled precursors, they tested for increased nucleic acid synthesis associated with photoconidiation. At 5 h post light induction, there was a massive increase in nucleic acid synthesis in the conidial ring area when compared with the dark grown controls and this increased until 8 h. The addition of RNA synthesis inhibitors at 3, 5 and 7 h inhibited sporulation, but had little or no effect on growth. RNA synthesis inhibitors added after 7 h had no effect on conidiation. Protein synthesis inhibitors were also shown to inhibit sporulation (Betina & Zajacová, 1978b). Together these results clearly demonstrated that *de novo* transcription and translation was required during photoinduced conidiation from at least 3 h and up to 7 h post exposure. The authors concluded that the ability to conidiate in response to light was related to the metabolic rate of the hyphal cell rather than to the size of the colony or hyphal cell age. This conclusion was supported by Galun (1971), who demonstrated that competency in *T. viride* decreased from the colony margin inward.

Gressel and Galun (1967) observed a decrease in nucleic acid synthesis distal and proximal to the conidiation ring from 16 h post light-induction, which suggests intercellular signalling may be occurring. The area distal was unable to perceive a second light induction and the authors postulated this to be associated with the decrease in nucleic acid synthesis.

1.4.2.2 Photochemistry of the Light Response

Induction of conidiation in response to light in *Trichoderma* spp. falls within the "blue-light effects" in fungi. The most photoresponsive wavelengths (peaks) are within the near-ultraviolet (320-380 nm) and blue (380-500 nm) spectra (Betina & Farkaš, 1998). In *T. viride*, peaks have been reported at 365, 430 and 480 nm (Gressel & Hartmann, 1968) and at 320, 380, 430 and 480 nm (Kumagai & Oda, 1969). The variation in reported spectra is not unexpected given the variation observed in other aspects of conidiation.

A correlation exists between the light intensity and the exposure period which determine the level of conidiation. This correlation follows the Bunsen-Roscoe reciprocity law of 1863, which states that different length exposures which produce the same number of photons will produce the same level of response (Gutter, 1957; Gressel *et al.*, 1975; Horwitz *et al.*, 1990). A light intensity of 25% for 60 s duration resulted in the same conidial rate as 12.5% intensity for 120 s in *T. viride* (Galun, 1971). Increasing

the beam width from 0.1 mm to 0.5 mm without changing the light intensity and duration resulted in a change from zero to full conidiation (Galun, 1971). The author asserts that adjacent irradiated hyphae were connected with respect to perception of photoinduction. Horwitz *et al.* (1990) validated the Bunsen-Roscoe reciprocity in the nanosecond to minutes range in *T. atroviride* (the *T. harzianum* isolate used in this study has since been re-identified as *T. atroviride* [Kindermann *et al.*, 1998]). In addition, they demonstrated no difference between the kinetics of the photoresponse at 3°C compared with 26°C, which suggested no enzymatic processes were involved. Their results suggested that the inductive event was a simple first-order photobleaching reaction. Photobleaching reactions can also occur in the absence of oxygen. Gressel *et al.* (1975) demonstrated that if oxygen was briefly removed from *T. viride* cultures and a pulse of light given, then conidiation would begin when cultures were transferred back to the air, whereas, Gutter (1957) demonstrated light-induced conidiation was inhibited in hyphal cells which develop in the absence of oxygen. Together, these studies show a clear differentiation between the initial photoreactions and the development of conidiation and suggest photoconidiation is a two-step process whereby oxidative processes are a requirement for the developmental step. This is supported by the observation that cultures which were exposed to light at low temperatures, then kept in the cold for an extended period of time would conidiate upon return to normal incubation conditions (Gressel *et al.*, 1975).

1.4.2.3 Light-associated Circadian Rhythms

Morphogenesis in response to a light-induced circadian rhythm has been demonstrated in many filamentous fungi, however it is not entirely clear whether circadian influences play a role in *Trichoderma* conidiation. In response to cyclical changes in light, temperature and other environmental influences, organisms have adapted to anticipate daily changes through the evolution of circadian rhythms (Lakin-Thomas and Brody, 2004). These rhythms have been well characterised in the fungus *Neurospora crassa*, which conidiates in the dark in a circadian fashion following exposure to light. Betina and Zajacová (1978a) and Schrüfer and Lysek (1990) investigated whether *Trichoderma* cultures would also conidiate rhythmically following a light dose and concluded that no circadian rhythm was apparent and conidiation was strictly light-inducible.

Deitzer *et al.* (1988) explored rhythmic conidiation in *T. atroviride* (formerly *T. harzianum* [Kindermann *et al.*, 1988]) and a rhythmically conidiating mutant derivative,

but were unable to determine if it was circadian in nature. They hypothesised that the refractory period between which light doses are effective was due to an endogenous change in light sensitivity and that this was associated with a circadian rhythm. Under constant light, the wild-type cultures conidiated continuously in a radiating pattern following a 24 h initial delay. The mutant also conidiated continuously under constant light, but lacked the initial delay and the radial pattern. No conidiation occurred in the wild-type under constant dark, whereas the mutant produced well-defined concentric rings about 12 h apart. The addition of deoxycholate to the medium slowed the growth rate of both the wild-type and the mutant and caused the wild-type to conidiate in rings under constant light. The rings were produced with circadian-like periodicity and the period of the rings produced by the mutant under constant darkness became circadian-like also. Using the deoxycholate medium, they demonstrated subtle rhythmic changes to light sensitivity, as measured by conidiation, in wild-type cultures grown in the dark and this had a period of about 27 h. The use of this detergent to increase the period length in the mutant and the observation of both the light sensitivity and banding pattern in dark-grown cultures would suggest these rhythms not to be circadian or light induced. However the authors do raise the possibility that deoxycholate may affect circadian rhythms.

1.4.2.4 Interactions between Nitrogen Status and Light Induction

It is commonly known that there is an interactive effect between nutrient and light stimulation of conidiation and indeed it is standard practice to use half strength PDA and light to stimulate conidiation for the regular maintenance of *Trichoderma* cultures (Kirstin McLean, pers. comm.). Ellison *et al.* (1981) demonstrated a link between nitrogen and light-induced conidiation. In a study looking at over forty *T. viride* soil isolates, half sporulated at the periphery and did not produce concentric rings when grown on PDA under natural light (Ellison *et al.*, 1981). Sporulation still required light, however exposure prior to reaching the edge of the plate had no effect. The same effect was observed when cultures confronted a physical barrier such as thick slides, suggesting that substantial arresting of forward growth of mycelium was necessary for induction in these isolates. Repetition of the same experiments on richer media resulted in concentric circles. By supplementing PDA with single nutrients present in the richer media, they demonstrated that addition of primary forms of nitrogen was able to convert a peripheral sporulator to a concentric one. They concluded that the uptake of nitrogen in a cell must be sufficient to allow both simultaneous growth and sporulation, which

suggested that isolates exhibiting peripheral conidiation were unable to uptake or assimilate nitrogen to the same level as those exhibiting concentric conidiation. In contrast, Schrüfer and Lysek (1990) were unable to convert sporulation phenotypes in their *T. viride* isolates through increasing available nitrogen. Their experiments also showed that hyphal elongation must be arrested for induction, however, they suggested that both light and physical barriers act to cease elongation. It should be noted that no reliable identification of the *T. viride* isolates was provided.

1.4.3 Injury-induced Conidiation

In addition to abiotic factors and light, mycelial injury has been demonstrated to induce conidiation in *T. atroviride* (Casas-Flores *et al.*, 2004). This was inadvertently discovered during a mutational study, in which key genes involved in mediating blue-light responses were knocked out. These mutants were unable to respond to light, but conidiated wherever the mutant cultures were injured by a scalpel during growth. The authors further investigated this mechanism of induction in the wild-type and again observed conidiation in response to injury. Cultures were grown in total darkness and injured with a scalpel under safe red light.

1.5 Regulation of Photoconidiation

1.5.1 BLR-1 and BLR-2

Blue-light induced conidiation in *T. atroviride* is mediated by the Blue Light Regulators, BLR-1 and BLR-2. *blr-1* and *blr-2* are orthologous to the well characterised *wc-1* and *wc-2*, and in *N. crassa*, WC-1 and WC-2 (White Collar 1 and White Collar 2) perceive and transduce all known blue light responses (Herrera-Estrella & Horwitz, 2007). Loss of either *blr-1* or *blr-2* in *T. atroviride* resulted in mutants unable to conidiate in response to light but able to conidiate in response to starvation and mycelial injury (Casas-Flores *et al.*, 2004). Loss of function *wc-1* and *wc-2* mutants are blind for all light-induced phenomena, however like *T. atroviride*, conidiation can be stimulated via nutrient deprivation (Ballario *et al.*, 1996; Linden & Macino, 1997). These studies demonstrated a clear separation between light perception and conidiation and suggested that the different signalling pathways stimulating conidiation merge at a step prior to the development of conidia. In addition to photoconidiation, BLR-1 and BLR-2 were shown to play a role in mycelial growth and glucose sensing of *T. atroviride* and this is both light-dependent and independent (Casas-Flores *et al.*, 2004; 2006).

1.5.1.1 Protein Structure

The protein structure of the BLR proteins is highly similar to the *N. crassa* WC proteins, which act as both photosensors and transcription factors. Both BLR-1 and BLR-2 contain conserved Per-ARNT-Sim (PAS) domains, three in BLR-1 and one in BLR-2 (Casas-Flores *et al.*, 2004). PAS domains have been shown to be involved in signal transduction through monitoring of the energy state of the cell, in protein-protein interactions and in the sensing of environmental signals (Taylor & Zhulin, 1999). In *N. crassa*, they have been demonstrated to be involved in the formation of the functional WC-1/WC-2 complex. The N-terminal PAS domain of BLR-1 is of the LOV-type (Light, Oxygen and Voltage) which, in WC-1, binds a flavin chromophore (flavin adenine dinucleotide [FAD]) allowing WC-1 to act as the blue-light photoreceptor (Herrera-Estrella & Horwitz, 2007). A single conserved GATA-like zinc-finger DNA-binding domain is also present in both BLR-1 and BLR-2, and BLR-1 possesses a nuclear localisation signal (NLS), which is conserved in position with WC-1. In WC-1, the NLS domain has been shown to be involved in binding to the promoters of light-regulated genes, demonstrating WC-1 to also act as a transcription factor. It is likely that BLR-1 and BLR-2 function in a similar fashion to WC-1 and WC-2, though some differences are apparent. Polyglutamine tracts at the N- and C-termini of WC-1 act as activation domains, however these are absent in BLR-1, suggesting an unidentified motif may act as the activation domain or this function may be accomplished by association with an activator protein (Herrera-Estrella & Horwitz, 2007).

In *N. crassa*, the WC-1 and WC-2 proteins together with FRQ (Frequency) are the key regulators of circadian photoconidiation, where WC-1 and WC-2 regulate conidiation and FRQ acts as the oscillator (Loros & Dunlap, 2001; Lakin-Thomas & Brody, 2004). This circadian clock has been the subject of much research, however it is evident from the literature that much is still unknown. Rhythms which do not require the WC proteins but respond to blue-light have been observed, suggesting additional blue-light receptors. Endogenous rhythms have also been identified and these may be linked to metabolism. No conclusive evidence of a circadian rhythm in *Trichoderma* spp. has been presented, however rhythmic light sensitivity has been observed and a mutant created which conidiates in a banding pattern in the dark (Deitzer *et al.*, 1988). It is not known whether BLR-1 and BLR-2 are involved in circadian responses in *Trichoderma* spp..

1.5.1.2 Light-dependent Gene Regulation by BLR-1/BLR-2

At least 40 genes have been shown to be regulated by BLR-1/BLR-2 and, in contrast to WC-1 and WC-2, negative regulation of gene expression by these proteins has also been demonstrated (Rosales-Saavedra *et al.*, 2006). The 40 genes exhibited changes in expression 1.8 fold or greater in response to light and were derived from a larger subset of light affected transcripts. In the wild-type, thirty genes were upregulated within 5 min following a brief pulse of blue-light and this expression was abolished in both Δ*blr-1* and Δ*blr-2*. Conversely, ten genes constitutively expressed in the dark were down-regulated in the wild-type following a light exposure and this repression was lost in the Δ*blr-1* and Δ*blr-2*. Studies on differential gene expression have identified many genes up-regulated during the blue-light response, which are under the control of WC-1/WC-2, however, only two of the forty genes analysed in *T. atroviride* have been previously demonstrated to be up-regulated by WC-1/WC-2 in *N. crassa* (Rosales-Saavedra *et al.*, 2006).

It is likely that some of the genes regulated by BLR-1/BLR-2 are involved in processes other than conidiation and indeed, *phr1* a DNA photolyase gene was present within the thirty up-regulated genes (Rosales-Saavedra *et al.*, 2006). DNA photolyases are flavoproteins that repair UV-damage and it is postulated that their induction in response to blue-light is in preparation for more damaging rays. This gene was identified previously in *T. atroviride* and demonstrated to be induced rapidly in response to blue light (Berrocal-Tito *et al.*, 1999; 2000). Interestingly, *phr1* transcripts could be detected across the whole colony following a light exposure suggesting all cells were sensitive to the light, whereas conidiation occurred only in a ring at the edge of the colony in these cultures, suggesting that light perception and conidial development are separate functions. This is supported by the observation that the photoreactions can occur in conditions inhibitive to conidiation (Gutter, 1957; Gressel *et al.*, 1975; Horwitz *et al.*, 1990; section 1.4.2.2). The *phr1* transcript level decayed slowly and in induced-cultures, expression could be detected in both mycelium and developing conidiophores (Berrocal-Tito *et al.*, 1999; 2000). The level was higher in conidiophores suggesting regulation of *phr1* is induced in the dark in association with conidial development. This is further supported by the fact that atropine inhibited photoconidiation but did not block *phr1* transcription in response to light (Casas-Flores *et al.*, 2006). The authors acknowledge that it is possible that atropine blocks conidiation at a later stage, however they propose that atropine may block an additional light input.

Analysis of the promoter regions of *phr1* and other rapid blue-light inducible genes in *Trichoderma* spp. has revealed the presence of conserved light-responsive elements (LREs), which supports a role for these in light-regulation. In *N. crassa*, it has been demonstrated that the light-activated WC-1/WC-2 complex interacts with the promoters of early light-inducible genes at the consensus LRE sequence GATNC----CGATN (He & Liu, 2005). Rosales-Saavedra *et al.* (2006) found LRE elements in most of the light responsive genes from their microarray study and at least one perfect consensus sequence was found in the promoters of all the BLR-dependent genes. These analyses support a role for this element in light-associated regulation and suggest most of the light-responsive genes are under the control of BLR-1/BLR-2.

1.5.1.3 Nitrogen Regulation of Conidiation

In *N. crassa*, many WC-1/WC-2 blue-light inducible genes have been shown to be induced by nitrogen starvation independently of WC-1/WC-2, which suggested these genes to be under the influence of nitrogen catabolite repression (Sokolovsky *et al.*, 1992). It is not known if BLR regulated genes are also under this control, however sudden nitrogen deprivation has been shown to stimulate conidiation in *T. atroviride* and this is independent of BLR-1/BLR-2 (Casas-Flores *et al.*, 2006).

1.5.1.4 Photoadaptation of BLR-1/BLR-2 Inducible Gene Expression

Many of the *T. atroviride* BLR-1/BLR-2-regulated, blue-light inducible, genes were down-regulated in constant blue-light, which is a phenomenon known as photoadaptation. Transcript levels of seven genes up regulated by BLR-1/BLR-2 were shown to start increasing after 5 min constant blue light, reaching a maximum after 30-60 min and decreasing after 240 min (Rosales-Saavedra *et al.*, 2006). Photoadaptation has also been observed with *phr1* transcript levels, suggesting this to be a common feature of rapid blue-light inducible genes in *T. atroviride*.

In *N. crassa*, VIVID, a small PAS/LOV domain protein, acts as the blue-light photoreceptor for photoadaptative responses (Schwerdtfeger & Linden, 2001; 2003). In addition to transient expression, VIVID has been demonstrated to be involved in regulating the 2 h refractory period before a second light exposure is effective in *N. crassa* (Schwerdtfeger & Linden, 2001). A refractory period between which light doses are effective has also been demonstrated in *T. atroviride* (Gressel & Galun, 1967; Deitzer *et al.*, 1988). An orthologue of VIVID, designated ENVOY, has been found in

Hypocrea jecorina (anamorph *T. reesei*). Though there was a high degree of similarity between the proteins, *env1* was unable to complement a *vvd* mutant strain (Schmoll *et al.*, 2005). It remains to be determined whether this protein is involved in photoadaptation in *Trichoderma* spp. (Rosales-Saavedra *et al.*, 2006).

Protein kinase C (PKC) has also been shown to play a role in photoadaptation in *N. crassa* through modulating the activity of WC-1. Members of the PKC family are Ca^{2+}/phospholipid-dependent serine/threonine protein kinases which act as transducers in the phosphoinositide cascade regulating a variety of cellular responses in eukaryotes including photoadaptation (Lendenfeld & Kubicek, 1998). In *N. crassa*, PKC has been shown to interact directly with WC-1 in dark-grown and light-adapted cells and to phosphorylate *in vitro* the zinc finger domain (Arpaia *et al.*, 1999; Franchi *et al.*, 2005). The authors postulated that PKC acts as a negative regulator of the WC-1/WC-2 complex by phosphorylating WC-1, thus modulating its activity. A PKC orthologue has been isolated from *H. jecorina*, however it is not known if this protein plays a role in photoadaptation in *Trichoderma* spp. (Lendenfeld & Kubicek, 1998).

1.5.2 BLR-1/BLR-2 and ENVOY: Mycelial Growth and Carbon Sensing

In *T. atroviride*, BLR-1/BLR-2 have been demonstrated to influence mycelial growth and carbon sensing, both of which have been suggested as roles for ENVOY in *H. jecorina*. Growth inhibition by constant blue light was more pronounced in *T. atroviride* Δ*blr-1* and Δ*blr-2* mutants than the wild-type, which suggested a light protective role for these regulators (Casas-Flores *et al.*, 2004). This phenotypic difference was also observed in a *H. jecorina env1* mutant in which the PAS domain had been removed ($env1^{PAS-}$), though the effect was more severe (Schmoll *et al.*, 2005). The authors suggested that this protection may involve DNA photolyase activity. In *T. atroviride phr1* is up-regulated in response to light and this is dependent on BLR-1/BLR-2 (Casas-Flores *et al.*, 2004). Schmoll *et al.* (2005) suggested that in *H. jecorina*, *phr1* regulation may also involve ENVOY.

BLR-1/BLR-2 and ENVOY have been implicated in carbon sensing, demonstrating a link between components of the light and nutrient sensing pathways in *Trichoderma*. In *T. atroviride* dark-grown cultures, sudden glucose deprivation triggers a conidial ring at the perimeter of the colony, whereas no response was observed in Δ*blr-1* and Δ*blr-2* mutants (Casas-Flores *et al.*, 2006). This suggested a light-independent role for BLR-

1/BLR-2 in carbon deprivation-induced conidiation. In *H. jecorina*, the addition of cellulose into the medium stimulates expression of the major cellobiohydrolase gene, *cbhI*. In constant light, cellulose-induced expression of *cbhI* is enhanced compared to that in constant darkness, whereas expression of *cbhI* was delayed under constant light in the *env1*$^{PAS-}$ mutant and, in constant darkness, *cbhI* was only transiently up-regulated in response to cellulose. These results suggested both light-dependent and independent roles in cellulose sensing (Schmoll *et al.*, 2005).

1.5.3 Novel Blue-light Photoreceptors

Research in both *Trichoderma* spp. and *N. crassa* has suggested the presence of other blue-light receptors and pathways, which may cross regulate the BLR-1/BLR-2 WC-1/WC-2 pathways. In *T. atroviride*, two blue-light up-regulated and one blue-light down-regulated gene were shown to respond to light independently of BLR-1/BLR-2. In *N. crassa*, components of the light-regulated circadian pathway have been shown to be cross-regulated by an unknown blue-light pathway (Rosales-Saavedra *et al.*, 2006; Lakin-Thomas & Brody, 2004). The WC orthologues, VIVID and ENVOY are the only blue-light photoreceptors identified to date in fungi. Orthologues of cryptochromes and dash-cryptochromes have also been identified in fungi, although no phenotypic effects have been observed in the corresponding mutants (Alfredo Herrera-Estrella, pers. comm.).

Complementation analysis of *T. atroviride* (*T. harzianum*) photoconidiation mutants suggested that 51-86 genes are essential for normal conidiation in response to light (Horwitz *et al.*, 1985a). Of the 86 photoconidiation mutants, 17 were defective in photoinduction but still able to conidiate in response to stress. These 17 mutants, specifically deficient in photoinduction, are called *dim* mutants (Horwitz *et al.*, 1985a). The absorbance spectra of intact colony sections were altered in two *dimY* mutants compared to the wild-type and a yellow substance was present within the culture media (Horwitz *et al.*, 1985a, 1985b). The authors believed that the *dimY* mutants were lacking or had a defective photoreceptor and the yellow substance was hypothesised to be the build up of a precursor. The DNA photolyase gene is induced in response to light by the photoreceptor transcription factors BLR-1/BLR-2 (Casas-Flores *et al.*, 2004). No difference in wavelength response peaks for photorepair and light-induced expression of *phr1* was observed in the *dimY* mutants compared with the wild-type (Sametz-Baron *et al.*, 1997; Berrocal-Tito *et al.*, 1999), whereas in Δ*blr-1* and Δ*blr-2* mutants both

expression of *phr1* and photoinduction of conidiation were abolished (Casas-Flores *et al.*, 2004). Together these results suggest that photoperception mediated by BLR-1/BLR-2 still occurs in the *dimY* mutants and the corresponding *dimY* genes act downstream of the initial photoperception reactions. The identity of the *dimY* genes remains unknown.

1.5.4 Signal Transduction Cascades

Organisms respond to environmental cues through a complex regulatory network of signal transduction pathways, which interact with each other in a tightly-regulated fashion dependent on extra- and intracellular conditions. Both cAMP and MAP-kinase signalling cascades play central roles in morphological and physiological changes in fungi associated with both external and internal cues (Lee *et al.*, 2003; Xu, 2000). Research into the biochemistry of photoconidiation and the discovery of *blr-1* and *blr-2* has revealed links between light induction and multiple signalling cascades within the hyphal cell. This has enabled the formation of preliminary models on the networks associated with photoconidiation and has given insight into the signalling pathways leading from blue light perception at the cell surface, to alterations in gene expression in the nucleus and on to the initiation of conidiation.

1.5.4.1 Rapid Biochemical Changes Associated with Conidiation

Multiple biochemical changes have been shown to occur within *T. viride* hyphal cells in response to light. These biochemical responses include changes in the membrane potential, intracellular acidification, a transient rise in ATP production and a biphasic rise in cAMP (Nemcovic & Farkaš, 1998; Grešik *et al.*, 1988; Grešik *et al.*, 1991). It has been suggested that these biochemical responses are part of the same physiological response; however the exact order of events is uncertain. Grešik *et al*. (1991) put forward the following hypothesis regarding the biochemical changes associated with exposure to light: In *T. viride*, light triggers the opening of K^+ channels in the cellular membrane, which results in an efflux of K^+ ions into the outer-membrane space causing hyperpolarisation. Light also stimulates an oxidative burst in the cell and H^+ ions are released from the light-induced mitochondria into the cytoplasm to compensate for the charge loss resulting in intercellular acidification, which depolarises the membrane. The H^+ gradient established across the mitochondrial membrane drives ATP production (Sulová *et al.*, 1990; Mitchell, 1961). Both increased ATP production and intracellular acidification have been shown to activate adenylate cyclase (Caspani *et al*, 1985; Pall,

1977; Rosenberg & Pall, 1983; Thevelein *et al*, 1987), which suggested a mechanism by which light stimulates cAMP production (Grešik *et al.*, 1988). Sulová *et al.* (1990) postulated that the energy released from the initial photoreactions drives the oxidative burst and further, that this is likely flavin-mediated. It is not known whether BLR-1 participates in these reactions.

1.5.4.2 cAMP Regulation of Conidiation

cAMP acts as a secondary messenger for morphogenic signals in both prokaryotes and eukaryotes and in species of *Trichoderma* it has been shown to influence conidiation. cAMP levels were shown to rise transiently during photoconidiation in *T. viride* and photoconidiation was significantly increased when grown in the presence of an inhibitor of cAMP degradation (Grešík *et al.*, 1988; Sulová & Farkaš, 1991). In addition, exogenous cAMP stimulated conidiation in both light and dark cultures of *T. viride*, (Nemcovic and Farkaš, 1998). In prokaryotes cAMP acts as a starvation signal, with intracellular levels low when glucose levels are high. The effect of exogenous cAMP has been shown to be more pronounced when cultures were grown on glucose-rich medium, which suggested that cAMP acts as a starvation signal in *T. viride.* This is supported by recent molecular studies in *T. virens* on the adenylate cyclase gene *tac1*. Adenylate cyclase catalyses the formation of cAMP from ATP and is therefore essential for cAMP production (Lee *et al.*, 2003). Knockout mutants of *tac1* in *T. virens* were unable to conidiate in the dark, which clearly demonstrated that starvation induction in the absence of light is cAMP-dependent (Mukherjee *et al.*, 2007). Interestingly, conidiation in the light still occurred in *Δtac1* mutants, which suggests that while photoconidiation is promoted by cAMP it is not dependent on it.

1.5.4.3 Cross Regulation: cAMP and Protein Kinase A

Combined molecular and biochemical studies have revealed regulatory interactions between the cAMP signalling pathway and blue-light responses in *T. atroviride*. As described previously (1.5.2), sudden carbon deprivation induced a ring of conidiation in *T. atroviride* wild-type but not in Δ*blr-1* and Δ*blr-2*, which demonstrated this response to require an active BLR pathway (Casas-Flores *et al.*, 2006). When mutant cultures were transferred to medium without glucose and containing cAMP the wild-type phenotype was restored, which suggested cAMP was involved in the BLR-associated carbon response.

The physiological effects of cAMP in fungi are mediated through PKA, a cAMP-dependent protein kinase (Lee *et al.*, 2003). cAMP binds to the PKA regulatory subunit which releases the catalytic subunit, thus activating its kinase activity. In *T. atroviride* a rise in PKA activity levels was detected within 5 minutes of light exposure and this was independent of BLR-1/BLR-2, which suggested PKA activity was induced via a novel blue-light pathway (Casas-Flores *et al.*, 2006). The authors further investigated the role of PKA through mutations in the PKA regulatory subunit gene *pkr-1*. PKA activity was constitutively high in *pkr-1* antisense mutants and conidiation was not inducible in response to light or glucose deprivation. Some conidiation was observed in *pkr-1* antisense mutants when transferred to nil glucose and cAMP, however this conidiation was not in a ring but at the centre of the colony. The authors postulated the cAMP-induced conidiation is, in part, independent of PKA activity. In mutants where *pkr-1* was overexpressed, PKA activity was very low and cultures hypersporulated under all test conditions, particularly in response to light and cAMP. Though no conidiation occurred in the *pkr-1* antisense mutants, induction of BLR-regulated genes in response to light was still observed. In contrast, no induction of BLR-regulated genes in response to light was detected in the *pkr-1* overexpression mutants. These results clearly demonstrated cross-regulation of the BLR pathway by the cAMP-PKA pathway. In addition, the results suggested that high PKA activity is essential for the transcription of rapid BLR-inducible genes, whereas low PKA activity is required for conidial development. The authors postulate that this cross regulation may be due to phosphorylation of BLR-1 by PKA.

Following a light dose, WC-1 becomes hyperphosphorylated and this transient phosphorylation is essential for induction of rapid blue-light inducible genes (Schwerdtfeger & Linden, 2003). In dark-grown cells and light adapted cells, PKC is thought to phosphorylate WC-1, thus modulating its activity (Franchi *et al.*, 2005). Casas-Flores *et al.* (2006) suggested that PKA may be involved in the phosphorylation of BLR-1 following a light burst, thus stimulating transcription. Binding sites for both PKC and PKA are present in both WC-1 and BLR-1, which supports their assertion. Casas-Flores *et al.* (2006) have put forward a hypothetical model for the relationship between the cAMP-PKA and BLR pathways (Figure 1.3).

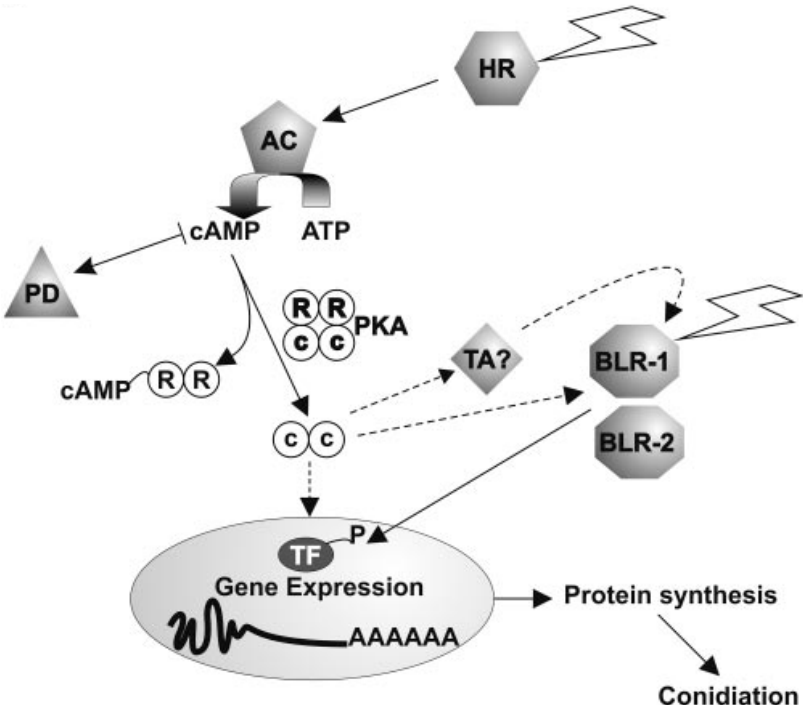


Figure 1.3. Hypothetical model proposed by Casas-Flores *et al.* (2006) describing the relationship between the cAMP-PKA and BLR pathways. Two independent light inputs are necessary to induce conidiation. The BLR independent pathway could activate adenylyl cyclase (AC), leading to the production cAMP, which in turn binds to the PKA regulatory subunit (R), resulting in the activation of the catalytic subunit (C). Phosphodiesterase (PD) would regulate the levels of cAMP, exerting a negative control on photoconidiation. The increase in PKA activity would activate the BLR complex, triggering the expression of the blue-light-responsive genes; such a function may involve direct phosphorylation of either of the BLR proteins or phosphorylation of an as-yet-unidentified regulatory partner (TA). Alternatively, PKA may phosphorylate a putative transcription factor (TF), whose modification is necessary for gene activation. Noncontinuous lines indicate hypothetical steps. Figure and text are from Casas-Flores *et al.* (2006).

1.5.4.4 Heterotrimeric G-proteins and MAP-kinase Cascades

G-proteins are an integral part of cell signalling and in fungi have been shown to be involved in sporulation, mating, pathogenicity, secondary metabolite production and vegetative incompatibility (Lee *et al.*, 2003). In *Trichoderma*, G-proteins have been shown to negatively regulate conidiation. Loss of the G-protein α-subunit gene (*tga1*) in *T. atroviride* resulted in intense conidiation. Conversely, *tga1* overexpression mutants were inhibited for conidiation (Rocha-Ramírez *et al.*, 2002). Loss of *tga3* also resulted in hypersporulation in *T. atroviride* and conidiation in the dark (Zeilinger *et al.*, 2005). The steady state levels of cAMP were reduced in *tga1* and *tga3* loss of function mutants, which was expected, however, addition of cAMP to the *tga3* mutant did not restore the wild-type phenotype. This result suggested that Tga3-mediated effects on

conidiation may be via a cAMP-independent pathway. In contrast, knockout mutations of *tgaA* (*tga1*) and *tgaB* in *T. virens* did not differ from the wild-type in conidial phenotype (Mukherjee *et al.*, 2004). It is also possible, as suggested by Casas-Flores *et al.* (2006), that exogenous cAMP acts differently, through a cAMP receptor.

Mitogen-activated protein kinase (MAPK) signalling cascades have been shown to regulate a variety of responses in the cell to do with growth, proliferation and virulence (Schaeffer & Weber, 1999; Xu, 2000). Loss of the MAPK gene, *tmk1*, from *T. virens* resulted in hypersporulation in the light and dark, similar to the G-protein mutants described previously (Mukherjee *et al.*, 2003), which suggested a repressive role for Tmk1 in conidiation.

1.6 Summary and Objectives

Conidiation in the ascomycetous fungi *Trichoderma* spp. is induced in response to light, injury and combinations of abiotic factors. Research into conidiation in *Trichoderma* spp. is extensive and ranges from applied studies examining the influence of abiotic factors to understanding the genetic basis of the light response. The identification of the blue-light regulators, *blr-1* and *blr-2*, and the association of the photoinduction pathway with cAMP and PKA have revealed great insights into the regulation of photoconidiation. Further studies will likely clarify the relationship between these pathways and the G-protein and MAP-kinase cascades implicated in conidiation.

Commercial preparation of the biocontrol fungus *Trichoderma* is achieved by mass production of asexual spores, or conidia, however anecdotal evidence suggests *Trichoderma* biocontrol agents (BCAs) are often poor sporulators, which favour mycelial growth instead. Mycelial growth is the active phase of the BCA, therefore cost effective production and efficient biocontrol requires a balance between reproductive and vegetative states of the fungus. The aim of this study was to increase knowledge on factors influencing and regulating conidiation in *Trichoderma*. Studies on conidiation have focused on two main species, *T. viride* and *T. atroviride*. In this study, multiple species will be compared to investigate if assumptions made on conidiation are generic to *Trichoderma*. Identification of key factors regulating or promoting conidiation should lead to recommendations for producers and users of *Trichoderma* BCAs.

The specific objectives of the first part of this study were to:

1. Evaluate the influence of nitrogen source and the ambient pH on light- and injury-induced conidiation
2. Investigate whether conidiation is associated with a circadian rhythm and provide molecular evidence of rhythmic gene expression in *Trichoderma*

Chapter Two
The Influence of Abiotic Factors on Conidiation

Trichoderma spp. reproduce asexually in response to nutrient deprivation, blue-light and mycelial injury. Much research has been undertaken into the optimal growth conditions for *in vitro* conidiation in many species of *Trichoderma* (section 1.4.1). Together, these studies showed that the carbon and nitrogen status and pH of the growth medium were the main nutritional factors influencing conidiation, however no single set of optimal parameters could be assigned to a particular species. Species-specific parameters may exist, however inadequate taxonomic identification of *Trichoderma* isolates has made it difficult to interpret the studies in the context of true species. None of the studies on the influence of nutrition and pH on conidiation in *Trichoderma* state that the isolates were identified by molecular methods, therefore, it is possible the identifications were incorrect. In older studies predating Bisset (1991) and, in particular, Rifai (1969), species identification was based on species-groups and individual species within a group were not recognised. The question of whether species-specific optimal conditions for conidiation exist remains largely unanswered.

The use of light to stimulate conidiation in *Trichoderma* spp. has allowed for precise studies in both photoinduction of conidiation and conidial morphogenesis. Photoconidiation research on isolates of *T. viride*, *T. atroviride* and *T. virens* demonstrated conidiation to be inducible by blue and/or UVA light (Section 1.4.2). In *T. atroviride*, this was shown to require the *blr-1* and *blr-2* genes, which are orthologues of *Neurospora crassa wc-1* and *wc-2* (Casas-Flores *et al.*, 2004; 2006; Section 1.5.1). In *Hypocrea jecorina* (*T. reesei*, anamorph), another putative blue-light receptor, *env1*, was identified with high similarity to *N. crassa vvd* (Schmoll *et al.*, 2005; section 1.5.1.4). Studies on *blr-1*, *blr-2* and *env1* in their respective hosts have demonstrated an interactive link between components of the light pathways and both carbon sensing and mycelial growth (Section 1.5.2).

Effects of light on metabolism have also been observed in other fungi, however the presence/absence of light within an experiment and the influence this may have on results is seldom considered (Schmoll *et al.*, 2005). In the nutritional studies on *Trichoderma* conidiation, the light conditions during incubation were either not

described, or the authors state that cultures were grown in the dark, but not whether they were protected from the light. For example, Jackson *et al.* (1991a) stated all cultures were grown in the dark, but rings of conidiation were clearly visible on the plates, which suggests that cultures were not protected from the light and daylight entered the incubator. It is not known whether photoinduction of conidiation is constant under variable nutritional conditions, therefore a lack of rigorous control on light exposure makes interpretation of the results difficult and invalidates comparison between studies.

The aim of this study was to gain a better understanding of conidiation in five biocontrol isolates representing separate species of *Trichoderma*. In contrast to previous studies, all isolates were identified using molecular methods and light was included as a controlled variable in all experiments. The objectives were to investigate light and injury induction of conidiation in a range of species in response to abiotic factors. Initially, the effects of different sources of nitrogen were investigated, which led to an examination of the role ambient pH plays in conidiation.

SECTION A
LIGHT- AND INJURY-INDUCED CONIDIATION

2.1 Introduction

A characteristic feature of *Trichoderma* species is the production of concentric conidiation rings in response to alternating light-dark cycles (Gutter, 1957). Indeed, exposing cultures of a certain age to blue light has been shown to be sufficient to produce a ring of profuse conidiation in isolates of *T. viride, T. atroviride*, and *T. virens* (section 1.4.2). This response has been shown to be due to blue and/or UVA light and, in *T. atroviride*, involves the regulatory elements BLR-1 and BLR-2 (Blue Light Regulators) (Casas-Flores *et al.*, 2004). In addition to light induction, conidiation is induced by starvation and more recently mycelial injury has been identified as a stimulus which acts independently of light and starvation in *T. atroviride*. Both a single light exposure and mycelial injury stimulate a highly ordered cascade of events culminating in conidiation approximately 24 h later, and are, thus, useful assays for studies on conidiation. In this section, conidiation in response to alternating light/dark conditions, a single blue-light exposure and mycelial injury was investigated in five biocontrol species (*T. hamatum, T. atroviride*, *T. asperellum* [formerly *T. viride*; Lieckfeldt *et al.*, 1999], *T. virens* and *T. harzianum*). Based on the literature and anecdotal observations from our laboratory, we hypothesised that all cultures would produce multiple conidiation rings under alternating light/dark conditions, a single ring of conidia in response to a discreet light burst and conidiate in response to mycelial injury in the absence of light.

2.2 Materials and Methods

2.2.1 Isolates

Five *Trichoderma* biocontrol isolates from New Zealand were obtained from the Lincoln University culture collection for use in this study (Table 2.1). All isolates had been previously identified on the basis of morphology and ITS sequencing (Kirstin McLean, pers. comm.). Isolates were maintained and stored on potato-dextrose agar (PDA) (Appendix 7.2.1.).

Table 2.1. *Trichoderma* isolates used in this study.

Species	Isolate	Origin	Isolation Date
T. hamatum	LU592	Horticultural soil, Christchurch, NZ	1997
T. atroviride	LU298	NZ	1989
T. asperellum	LU697	NZ	1989
T. virens	LU540	NZ	1992
T. harzianum	LU675	Willow tree, Hamilton, NZ	1963

2.2.2 Incubation under Alternating Light/Dark Conditions

The effect of alternating light/dark exposure on conidiation was examined on PDA medium for all isolates listed in Table 2.1. The PDA was sterilised in a laboratory stove top pressure cooker and poured into 150 mm Petri dishes. Three plates per isolate were inoculated centrally with 2 µL of a 10^8/mL conidial suspension, previously stored at -80°C in 20% glycerol (Appendix 7.2.2), and incubated unsealed at 23°C under 12/12 h alternating light/dark cycles, with standard white light, for 7 d. The experiment was repeated once.

2.2.3 Blue-light Photo-induction of Conidiation

Conidiation in response to a single light-dose was investigated as described by Gutter (1957). Photo-induction of conidiation was done in a purpose-built white wooden box (650 mm wide, 500 mm deep and 700 mm high), which was five-sided and the front covered by dense cloth material. A double fluorescent light fitting was hung from the top of the box and for all light assays, two blue light fluorescent tubes (L 18 W/67; Osram GmbH., Munich, Germany) were placed in the fittings. Most of the light energy was within 420 – 520 nm and the fluence rate at the bottom of the box was 43 µmol/m^2/sec. Culture plates were prepared as described in Section 2.2.2 of this thesis using standard 90 mm Petri dishes, except in the pilot assay where PDA was sterilised in an industrial autoclave. Plates were incubated unsealed at 25°C in total darkness for 48 h, then placed on the floor of the light-box and given 15 min blue light exposure and returned to the incubator for a further 72 h. Control plates were incubated in total darkness for the duration of the experiment. All observations and manipulations were done in a dark room under safe red-light illumination (Kodak [Australia] Pty., Ltd., Coburg, Vic., Australia). In initial pilot assays, four plates per treatment were inoculated. No variation between plates was observed so replicates were reduced to two for the main experiment and the entire experiment was repeated once.

2.2.4 Hyphal Injury

Injury-induced conidiation was essentially as described in Casas-Flores *et al.* (2004). Culture plates were prepared as described in section 2.2.2 using standard 90 mm Petri dishes, except in the pilot assay where PDA was sterilised in an industrial autoclave. Plates were incubated unsealed at 25°C in total darkness for 72 h, then cut 3-4 times in a vertical or both vertical and horizontal directions with a sterile scalpel under safe red light and incubated in total darkness for a further 72 h. Control plates received no injury and were incubated in total darkness for the duration of the experiment. Replication was as described in Section 2.2.3.

2.3 Results

2.3.1 Incubation under Alternating Light/Dark Conditions

In the first assay, five isolates of *Trichoderma* were cultured under what is considered typical laboratory conditions of 12 h light/12 h dark cycles. All isolates produced concentric rings of conidiation in response to the alternating light/dark conditions and conidial maturation proceeded from the centre outwards (Figure 2.1). Each ring represented the colony front on consecutive days and the number of rings varied between isolates due to differences in growth rate. In addition, there was a 2-3 d delay between light exposure and the appearance of each ring. No variation was observed between replicates and repeat experiments. A central disk and two concentric rings of conidiation were observed in *T. hamatum* and the rings were thin in comparison to the other isolates. Pigmentation was observed on the central disk and first ring only. If plates were incubated for an additional 7 d, all rings became pigmented. *Trichoderma atroviride* produced three thick rings of conidia and a possible fourth at the edge of the plate and these appeared even in distribution of conidia. Four rings were observed in *T. asperellum* with the inner three rings only pigmented. Unlike *T. hamatum* and *T. atroviride*, *Trichoderma asperellum* produced conidia between the rings and as a result the rings appeared to merge. Five rings were visible in *T. virens* and, of these, the inner four were pigmented. Like *T. asperellum*, the rings appeared to merge and, in addition, alternated with rings of aerial hyphae. In *T. harzianum*, four distinct rings were produced. Mature green conidia were observed within the inner three rings and the outer ring was partially pigmented. Some of the rings appeared uneven in conidial distribution as described for *T. asperellum.* A yellow pigment was excreted into the medium by *T. harzianum*.

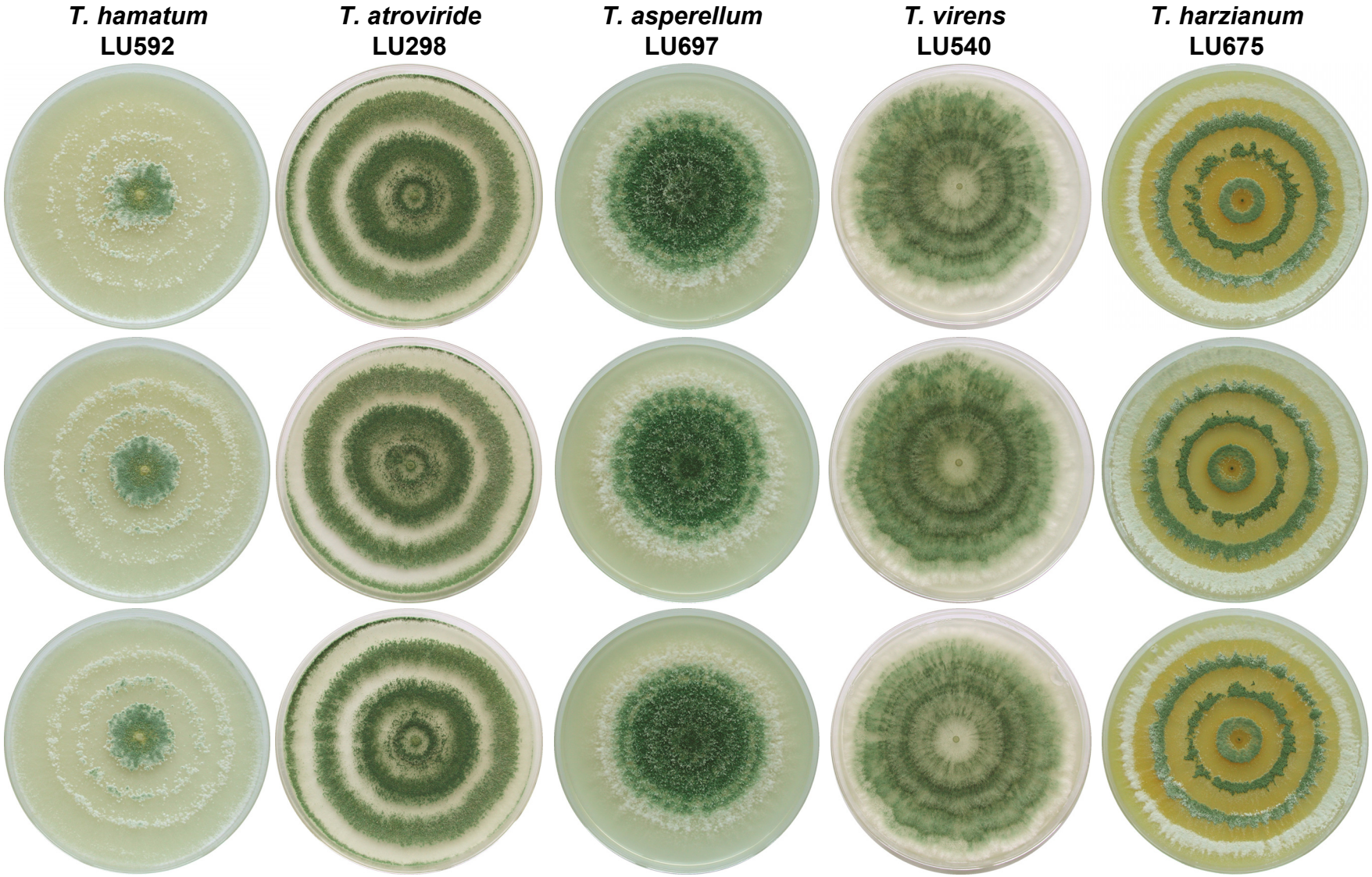


Figure 2.1. *Trichoderma* spp. grown under alternating light/dark conditions for 7 d. Three replicates are shown for each species.

2.3.2 Blue-light Photoinduction of Conidiation

The effect of a single blue-light exposure on conidiation was investigated in all *Trichoderma* isolates. The control plates were incubated in total darkness for the duration of the experiment (5 d; 120 h) to assess the level of conidiation occurring in the absence of stimuli. Conidiation did occur on the control plates for some isolates (Figure 2.2A). In control plates for all experiments, no conidiation was observed in either *T. hamatum* or *T. atroviride*. Some conidiation was observed in *T. asperellum* and this was localised to the centre of the plate. In both *T. virens* and *T. harzianum*, intense conidiation was apparent across the entire plate. In *T. virens*, pigmented conidia were observed on the surface of the plates but these were covered by a layer of aerial mycelium (Figure 2.2B). In *T. harzianum*, colourless to green pigmented conidia were present in a thick dense mat covering the plate. Some yellow/orange discolouration of the medium was also apparent underneath the colourless conidia at the centre of the plate, which indicated presence of the yellow pigment (2.2B).

The treatment plates received a single dose of blue light at 48 h when the colony diameters of all cultures ranged from 40 to 50 mm. For *T. hamatum*, sparse unpigmented conidia were observed clustered around the centre of the plate and the outer limit of this conidiation did not correlate with the margin of the colony at the time of light exposure (Figure 2.2C). In the repeat of the main experiment, the plates were incubated for a further 5 d at which time the conidia were observed to be pigmented green (data not shown). In *T. atroviride*, a broken ring of conidia was produced in response to light and the margin of this ring correlated with the position of the hyphal front at the time of light exposure. Sparse conidia were present back from the edge of the ring to the colony centre. This was referred to as a 'partially filled in' appearance. As with *T. hamatum*, if the cultures were allowed to incubate for a further 5 d, pigmentation eventually occurred. In response to light, *T. asperellum* produced a ring of pigmented spores which correlated with the position of the colony margin at the time of light exposure. Like *T. atroviride*, the ring appeared 'partially filled in' with unpigmented conidia. If incubated longer, these conidia became pigmented. *Trichoderma virens* also produced a ring of green pigmented conidia which correlated with the colony margin at the time of light exposure. This was in addition to the conidiation generally observed over the surface of the plate, which was evident on the controls. Like the controls, aerial mycelium was observed over the surface of the treatment plates. In contrast to the control plates, conidiation did not occur in *T.*

harzianum across the entire plate, rather conidiation was largely restricted to a central pale-green disk, the margins of which correlated with the colony margins at the time of light exposure. When left longer, this disk became darker green like that shown in Figure 2.1. A yellow pigment was also present in the medium as described for the controls. No variation was observed between the replicate plates and between experiments for both the control and treatment plates.

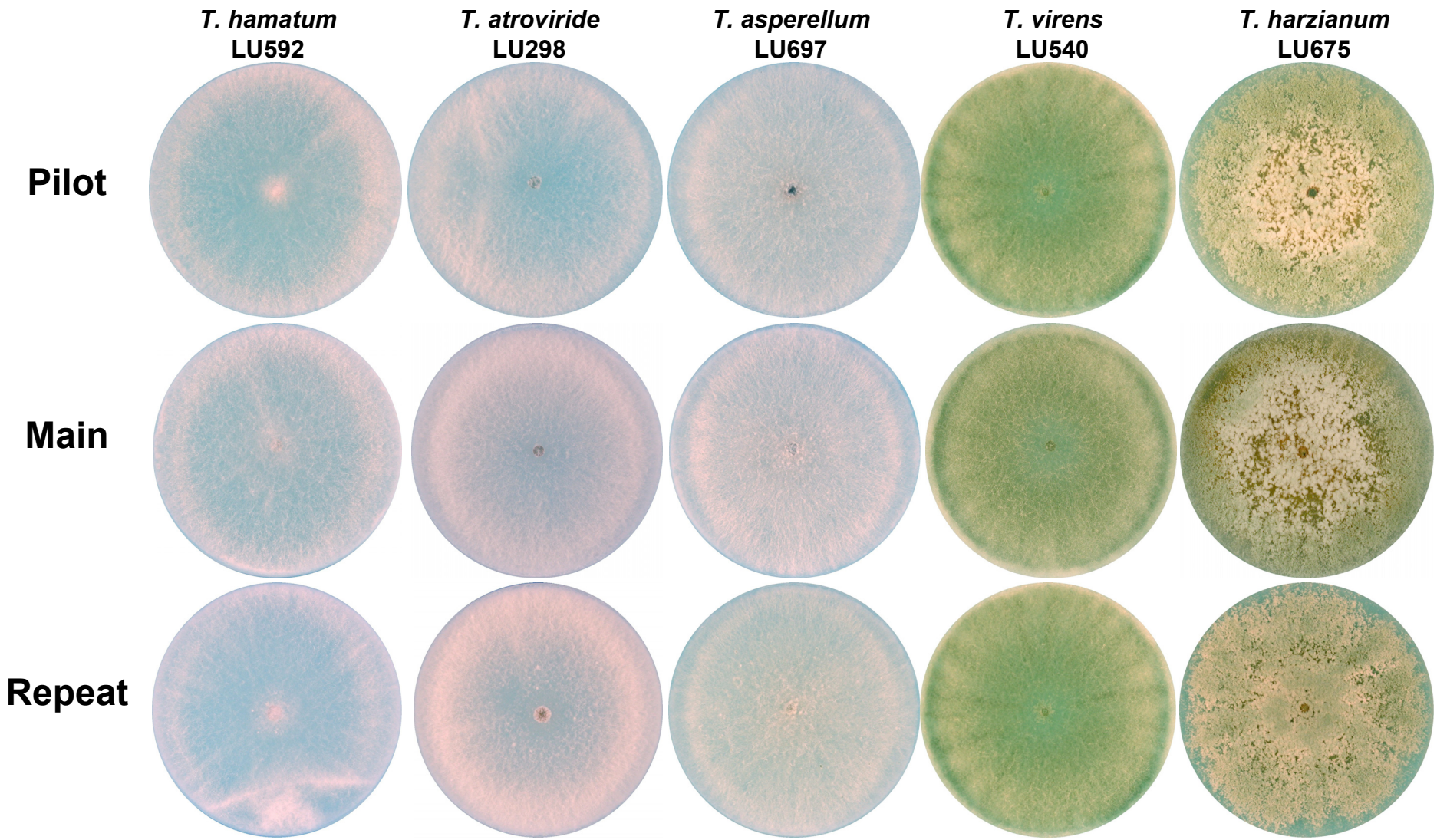


Figure 2.2A. *Trichoderma* photoconidiation control plates. Cultures were grown for 5 d in total darkness. One replicate each from the pilot assay, main experiment and repeat of the main experiment is presented. Plates were photographed on a blue background for contrast resulting in a pink tint in some images.

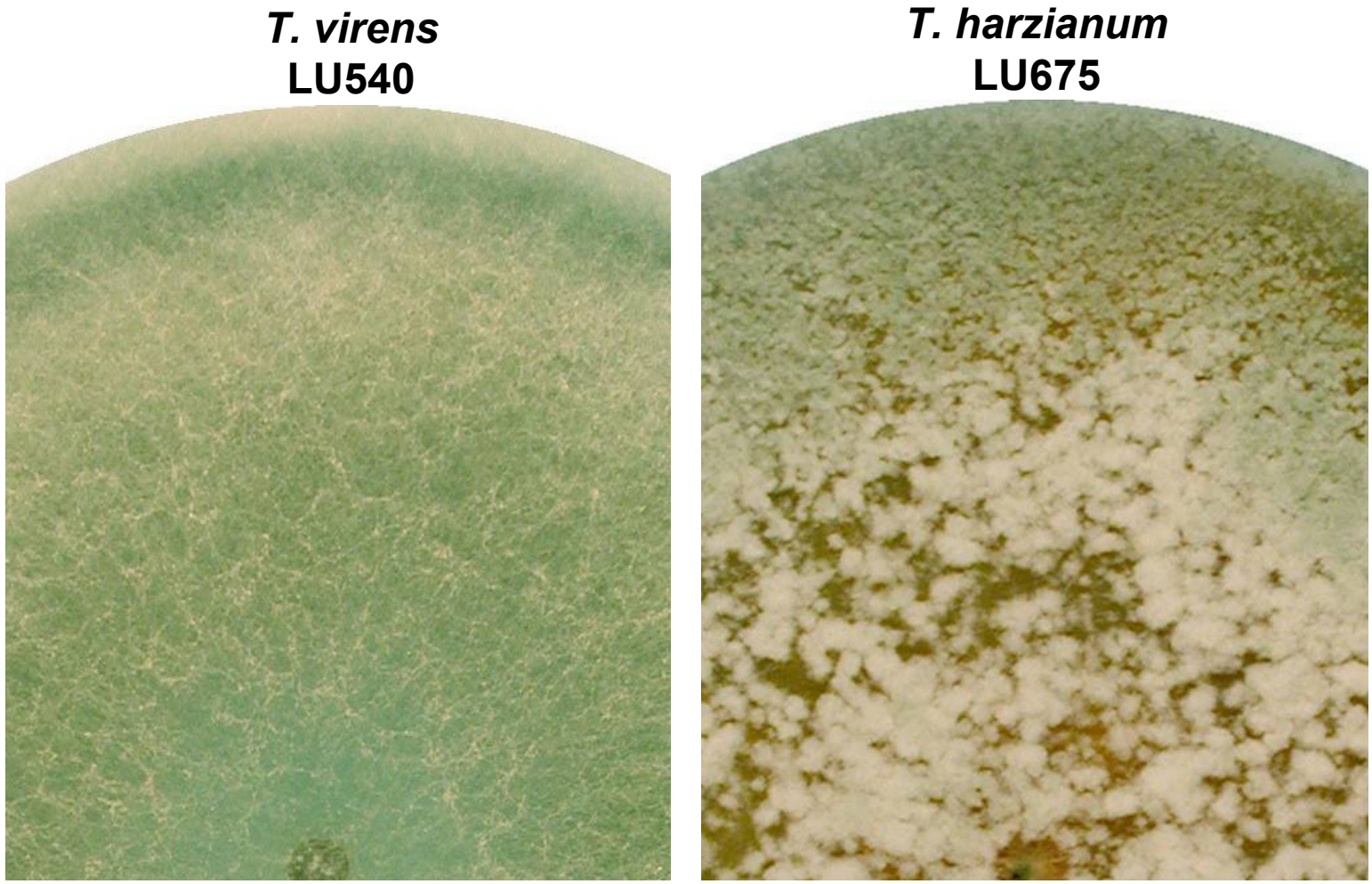


Figure 2.2B. Enlargement of the main experiment control plates for *T. virens* and *T. harzianum*.

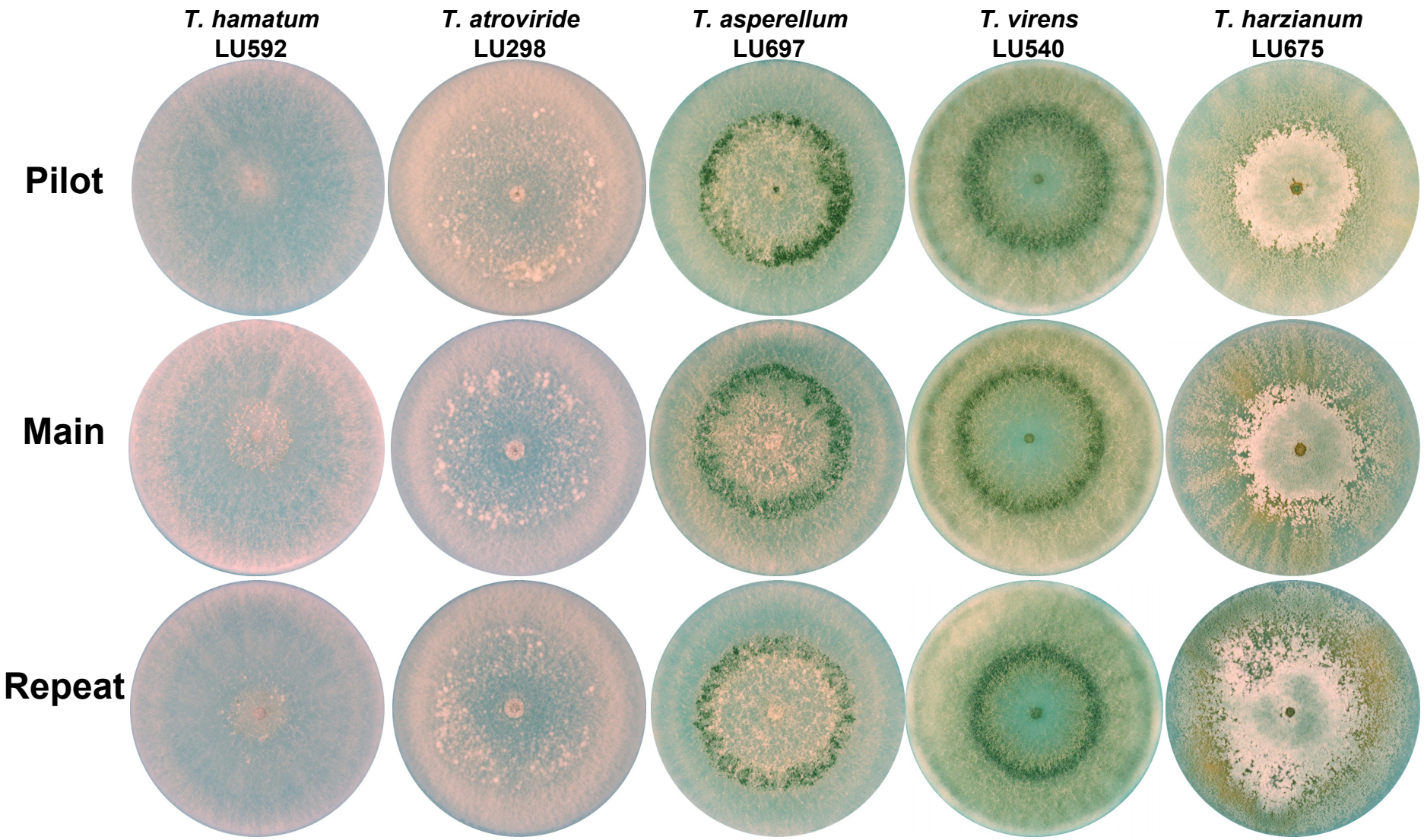


Figure 2.2C. *Trichoderma* photoconidiation treatment plates. Cultures were grown for 48 h in total darkness, exposed to blue-light for 15 min then grown for a further 72 h in total darkness. One replicate each from the pilot assay, main experiment and repeat of the main experiment is presented.

2.3.3 Induction of Conidiation by Hyphal Injury

Response to mycelial injury in the absence of a light stimulus was assessed for all *Trichoderma* isolates. As in Section 2.3.2, the control plates were incubated in total darkness for the duration of the experiment, which in this experiment was 6 d (144 h) rather than 5 d (120 h). No conidiation was apparent in *T. hamatum* cultures; whereas some conidiation was apparent in *T. atroviride*, though it was very sparse and varied between experiments (Figure 2.3A). Conidiation was also apparent across the *T. asperellum* plates and the density of conidiation varied between experiments. Conidiation was apparent on the surface of the *T. virens* plates and, as in the photoconidiation experiment, a layer of aerial mycelium was observed over the conidia, though more dense. A thick layer of conidia was observed on the *T. harzianum* plates and these appeared more pigmented than the controls shown for Section 2.3.2. The yellow pigment observed in previous experiments was also present. Though slight variation was apparent between experiments, no variation was observed between replicates within each experiment.

The treatment plates were also incubated for 6 d in total darkness, however at 72 h when the colony margins were 70 to 80 mm, the colonies were sliced with a scalpel to assess the response to mycelial injury. No conidiation at the site of injury was observed in *T. hamatum* (Figure 2.3B). Some unpigmented conidia were observed between the injury sites and conidiation was more concentrated in the repeated experiment where conidiation appeared constrained in a ring which represented the colony margin at the time of injury. Conidiation at the site of injury was observed with *T. atroviride* and the conidia were unpigmented except at the position of the colony margin when injured (Figure 2.3B). *Trichoderma asperellum* responded to injury in a similar fashion to *T. atroviride*, though conidia were evenly pigmented along the injury lines. Conidia were produced in response to injury in *T. virens* with pigmentation appearing more intense at the outer margins. Within the dense background conidial mat produced by *T. harzianum*, pigmented conidia were clearly formed in response to injury and a yellow pigment was excreted into the medium.

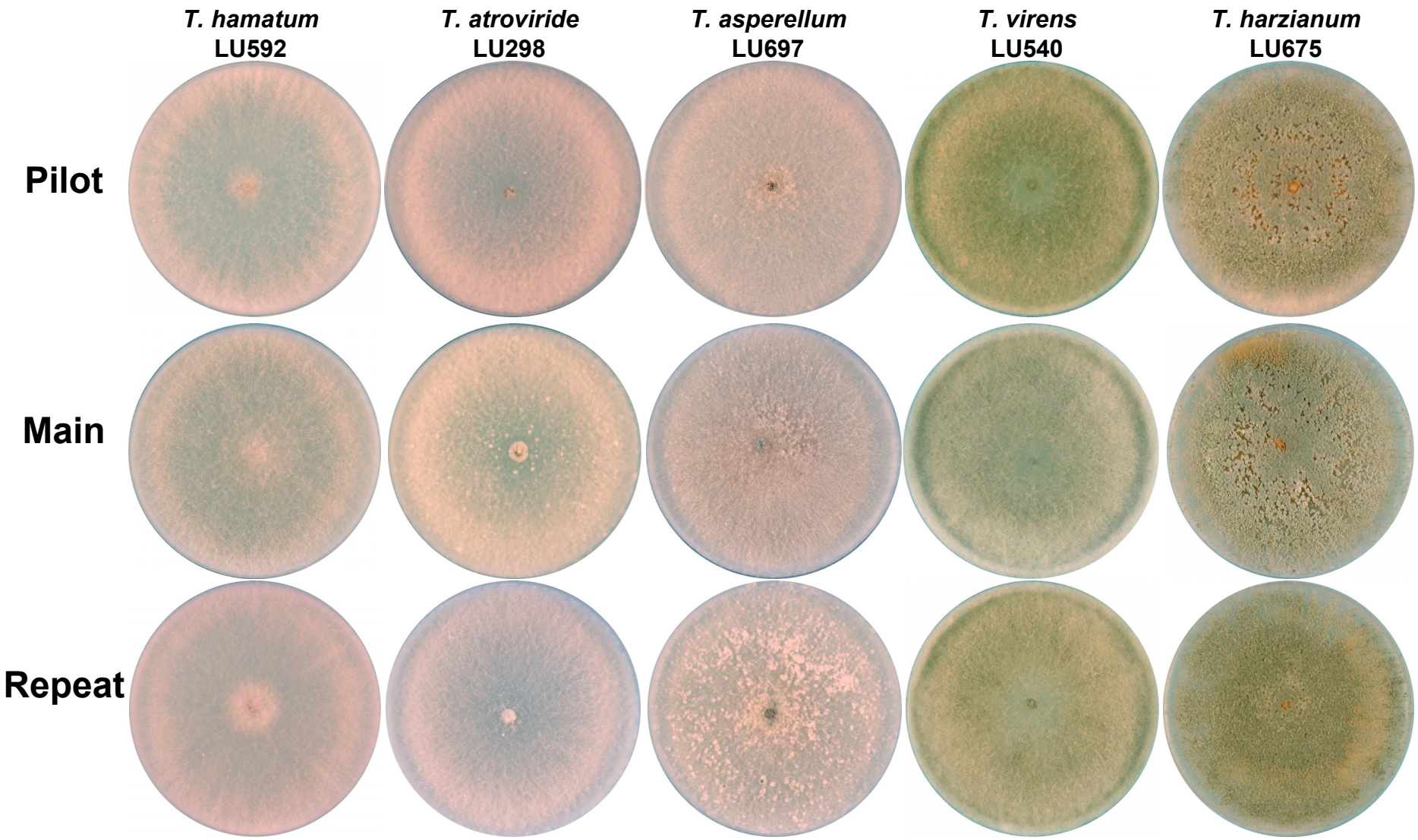


Figure 2.3A. *Trichoderma* injury induction control plates. Cultures were grown for 6 d in total darkness. One replicate each from the pilot assay, main experiment and repeat of the main experiment is presented.

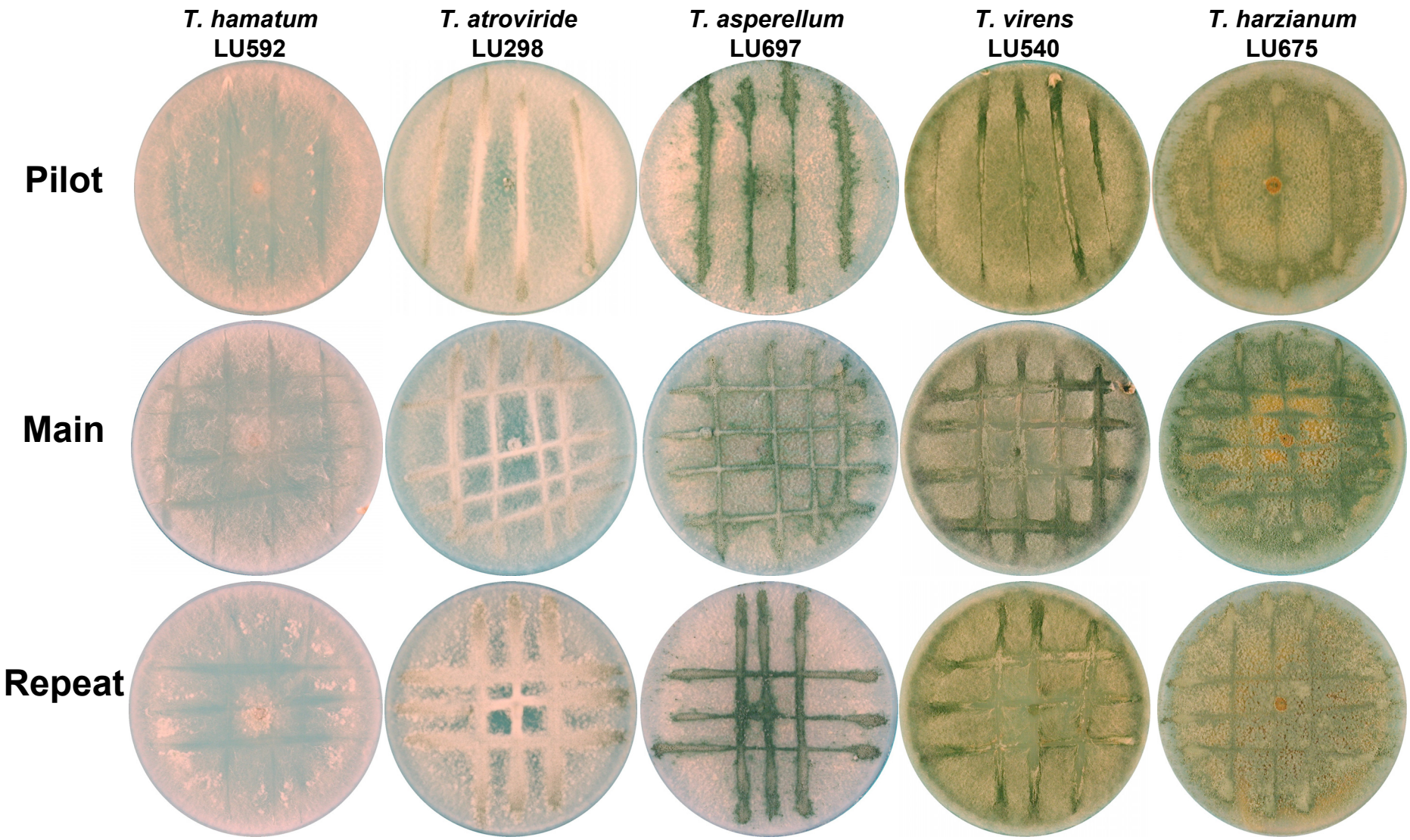


Figure 2.3B. *Trichoderma* injury induction treatment plates. Cultures were grown for 72 h in total darkness, then mycelium sliced with a scalpel under safe red-light and grown for a further 72 h in total darkness. One replicate each from the pilot assay, main experiment and repeat of the main experiment is presented.

2.4 Discussion

Conidiation in *Trichoderma* is a well studied phenomenon in *T. viride* and *T. atroviride*, which have dominated *Trichoderma* conidiation research and served as both the morphological and molecular models. In this study, conidiation was induced and observed in five biocontrol isolates of *Trichoderma* in three separate experiments. The isolates represented different species of *Trichoderma*, which had been identified on the basis of ITS sequence analysis, and included *T. asperellum* (formerly *T. viride* [Leickfeldt *et al.*, 1999]) and *T. atroviride* in addition to *T. hamatum*, *T. virens* and *T. harzianum*. In response to alternating light/dark conditions, all isolates produced multiple rings of conidia, however, contrary to our hypothesis, only three of the five isolates produced a single ring in response to a discrete light exposure and one isolate did not respond to injury. These results show that a single burst of light to a dark-grown *Trichoderma* spp. colony can induce either a ring or a disk of conidia and suggest that not all isolates of *Trichoderma* conidiate in response to injury. These findings demonstrate that the range of conidial responses in species of *Trichoderma* is greater than previously reported, and highlight the need to widen the study of conidiation across the *Trichoderma* genus rather than relying on just two species and indeed in some instances just two isolates.

Both *T. hamatum* and *T. harzianum* produced multiple rings of conidiation in response to alternating light/dark conditions, however, in response to a single light dose, a cluster of conidia at the centre and a large disk of conidia were produced, respectively, rather than the predicted single ring. These results were unexpected and challenge the current understanding of the photoconidial response in *Trichoderma*. The ability of a hyphal cell to undergo photoconidiation is referred to as competency and has been suggested to be due to the metabolic rate of the hyphal cell (Gutter, 1957; Gressel and Galun, 1967; Galun, 1971; Section 1.4.2.1). Typically, competency was greatest in the youngest cells in the colony, which is the outer colony margin. Under constant light conditions, conidiation was reported to occur across the colony, whereas in response to a brief pulse of light it occurred only at the perimeter. Presumably, under constant light conditions, all cells became competent when they passed through the responsive metabolic phase during development and growth. The above authors postulated that insensitivity to the light in older, and presumably, less metabolically active cells may be due to degradation of products required to transduce the light signal, however it is now known that hyphal cells behind the conidiation ring do respond to the light signal. The *T. atroviride* DNA

photolyase gene *phr1* is blue-light inducible and its expression has been detected across the colony following a light pulse (Berrocal-Tito *et al.*, 1999; 2000). Both *phr1* expression and photoconidiation require the Blue-Light Regulators BLR-1 and BLR-2, yet photoconidiation in these cultures occurred only at the perimeter (Casas-Flores *et al.*, 2004). In addition to demonstrating that light induction and conidial development are separate functions, it also suggests that competency relates to conidial development rather than photo-perception. This is further supported by the fact that addition of atropine blocks photoconidiation but it doesn't prevent induction of *phr1* (Casas-Flores *et al*., 2006)

In the *T. harzianum* isolate used in this study, conidiation occurred across the colony in response to a single light exposure, therefore all cells were competent at the time of light exposure. In addition, conidia were more profuse and mature in the centre of the disk compared with the perimeter suggesting competency decreased from the colony centre outwards. The *T. hamatum* isolate behaved in a similar fashion, however the response was significantly less intense overall and suggests this isolate to have a weak conidiation capability under these conditions. Interestingly, *T. hamatum* LU592 conidiates profusely under constant light (Appendix 7.3.1). If competency relates to metabolic activity, then in *T. harzianum* and *T. hamatum*, cells at the centre of the colony were more metabolically active than at the perimeter. It is standard laboratory practice to sub-culture only from the colony perimeter, as the youngest cells are the most rapidly growing (Moore, 1998). Therefore, it would seem highly unlikely that in *T. harzianum* and *T. hamatum*, the colony centres would be the most active. This phenotypic response is clearly different from those observed in other species and is difficult to reconcile with the current hypotheses on the theory of competency. Clearly it demonstrates that competency is not restricted to young cells and, therefore, competency is more likely connected to a metabolic state rather than the metabolic rate of the hyphal cell.

Conidiation in response to injury in the absence of additional stimuli has been described for *T. atroviride* (Casas-Flores *et al.*, 2004). In this study, the conidial injury response was described for three additional species: *T. asperellum*, *T. virens* and *T. harzianum*. No obvious conidiation was observed in *T. hamatum*, which suggests this isolate does not conidiate in response to injury, however, the conidiation capability of this isolate, as

seen in the single light exposure experiment, may have constrained the ability to detect a response.

There were clear differences between all isolates in the degree of conidiation in all experiments and this may reflect inherent metabolic differences between isolates (species). There was great variation in the conidial response to both light and injury stimuli between the isolates, however the response was consistent between replicates and experiments and each isolate could easily be discerned on the basis of conidiation pattern. Competency for photoconidiation is believed to be associated with cellular metabolism. The main factors influencing cellular metabolism are likely nutritional, therefore differences in competency between the isolates may reflect variation in each individual's ability to uptake and assimilate nutrients from the culture medium. Whilst it is not possible to determine from the current study, this variation may represent species-specific responses. The differences between the *T. atroviride* and *T. harzianum* isolates were striking, yet these species are closely related and are often taxonomically mistaken for the other. Studies in *T. viride* reported variation between isolates of *T. viride*, however recent taxonomic revision of this genus raises the possibility that the variation was due to species-specific differences. Gutter (1957) found two distinct competency morphologies in multiple isolates of *T. viride* and Ellison *et al.* (1981) and Schrüfer and Lysek (1990) incubated multiple isolates of *T. viride* under alternating light/dark cycles and observed two distinct morphologies. Leickfeldt *et al.* (1999) later revised this species and separated it into *T. viride* and *T. asperellum*, which suggests the two morphologies likely represented two species. Conidiation under alternating light/dark conditions in multiple isolates has been observed and reported for other *Trichoderma* species and invariably species-specific characteristics are observed, however, gross conidiation patterns are generally not considered reliable taxonomic markers on their own (Kullnig *et al.*, 2001). The greatest variation between isolates was observed in the single light dose and injury experiments. Using these two controlled experimental systems, it may be possible to identify reliable morphological taxonomic characters for the identification of *Trichoderma* species.

Conidiation on the control plates from both the photoconidiation and mycelial injury experiments occurred in the absence of light or injury stimuli and may be due to nutrient deprivation. In a closed system, growing fungal cultures rapidly remove the available carbon and nitrogen from the medium. At the same time, fungi sense and respond to the

depletion in the environment by relieving carbon and nitrogen catabolite repression, which in turn promotes metabolism of secondary substrates. Carbon and nitrogen deprivation are also known to induce conidiation in *Trichoderma*. In these experiments, all isolates except *T. hamatum* conidiated in the absence of light and the degree of conidiation in these plates varied greatly between isolates. If the conidiation observed in the absence of stimuli was due to nutrient depletion, then it is possible that *T. harzianum*, which conidiated extensively, depleted the available carbon and nitrogen at a faster rate or is more sensitive to nutrient depletion. Variation in metabolic activity was also postulated to be involved in the unusual photoconidiation phenotype of this *T. harzianum* isolate. An analysis of the carbon and nitrogen content of the medium would be required to address this theory.

Under all conditions tested in this section, the *T. harzianum* isolate secreted a yellow pigment into the medium. Though the identity of the pigment remains to be determined, yellow pigments have been observed previously in isolates of *T. harzianum* (Ghisalberti *et al.*, 1990; Vinale *et al.*, 2006) and *T. viride* (Betina, 1995) and identified as anthraquinones. Anthraquinones are secondary metabolites derived from polyketides and have been demonstrated to have variable antifungal activity (Ghisalberti & Sivasithamparam, 1991; Sivasithampararam & Ghilsalberti, 1998).

SECTION B
THE INFLUENCE OF PRIMARY AND SECONDARY NITROGEN ON LIGHT AND INJURY INDUCED CONIDIATION

2.5 Introduction

The intensity and distribution of conidia varied considerably between five isolates (species) of *Trichoderma*, in response to light or injury and, in the absence of stimuli (Section A). The underlying cause of this variation was unknown, however it was postulated to be due to differences between isolates in the metabolic activity in different parts of the culture. Carbon and nitrogen levels and the C:N ratio, in addition to the ambient pH, are considered to be the main environmental factors influencing conidiation in *Trichoderma* (Gao *et al.*, 2007; Chapter 1, Section 1.4.1). In the presence of preferred carbon or nitrogen sources, organisms repress expression of genes required for the utilisation of secondary sources and this is referred to as catabolite repression. Under carbon or nitrogen deprivation, or when primary sources are low and secondary sources are high, derepression occurs. In *T. atroviride* sudden carbon deprivation has been shown to induce a ring of conidia at the colony perimeter and this was dependent on expression of *blr-1* and *blr-2*, which clearly demonstrated a link between carbon sensing and the light-induction pathway (Casas-Flores *et al.*, 2006). Sudden nitrogen deprivation in *T. atroviride* resulted in a disk-like conidial response and this was independent of the BLR pathway, however studies in *N. crassa* have demonstrated cross-regulation of the photoconidiation pathway by nitrogen starvation. Nitrogen deprivation induced expression of blue-light inducible genes in *N. crassa* and this was independent of *wc-1*/*wc-2* (Sokolovsky *et al.*, 1992). These studies showed a clear interaction between catabolite repression and aspects of light-induction and conidiation. Amino or ammonium sources of nitrogen typically act as primary nitrogen sources in fungi, whereas nitrate forms are utilised as a secondary source. Glutamine is considered the most critical environmental nitrogen source in determining nitrogen catabolite repression and KNO_3 is an excellent inducer of derepression (Marzluf, 1997). In this section, cultures were subjected to light and injury stimuli in the presence of glutamine, urea or KNO_3. Urea was used as an ammonium nitrogen source and therefore alternate primary nitrogen source. Based on the literature and observations from the previous section, we hypothesise that light and injury will induce different conidiation

phenotypes in nitrogen catabolite repressed cultures compared with derepressed cultures.

2.6 Materials and Methods

2.6.1 Conidiation on pH-buffered Nitrogen-amended Minimal Medium Agar

Light- and injury-induced conidiation in the presence of primary and secondary nitrogen sources was investigated using all isolates listed in 2.2.1. Conidial suspensions, prepared as described in 2.2.2, were used to inoculate a pH-buffered minimal medium (MM) agar with 2% glucose and 25 mM L-glutamine or 25 mM urea or 50 mM KNO_3. MM agar was prepared as follows: 0.2 g $MgSO_4.7H_2O$, 0.9 g K_2HPO_4, 0.2 g KCl, 2 mg $FeSO_4.7H_2O$, 2 mg $ZnSO_4.7H_2O$ and 2 mg $MnCl_2.7H_2O$ were dissolved in 500 mL 2X pH 5.4 citrate/phosphate buffer (McIlvaine buffer, Appendix 7.2.3). Water was added to just under 1 L volume and the final pH adjusted to 5.4 with HCl. This solution was then mixed with 15 g agar prior to sterilisation. Media and amendments were sterilised in a laboratory stove top pressure cooker, except glucose which was filtered using a 0.45 μM filter. Nitrogen and glucose amendments were added to the MM agar after sterilisation. Plates were left unsealed and incubated at 25°C in total darkness and either photoinduced at 48 h or injured at 72 h, as described in 2.2.3 and 2.2.4, respectively. Two plates per treatment were inoculated and the entire experiment was repeated twice.

2.6.2 Conidiation on Unbuffered Nitrogen-amended Minimal Medium Agar

Light- and injury-induced conidiation were investigated on unbuffered MM agar, pH adjusted to 5.4 and amended with nitrogen, using all isolates listed in 2.2.1. Conidial suspensions, prepared as described in 2.2.2, were used to inoculate MM agar with 2% glucose and 25 mM L-Glutamine or 25 mM urea or 50 mM KNO_3. Media was prepared essentially as described in 2.6.1 except that all components except agar, glucose and nitrogen were dissolved in 500 mL water rather than citrate/phosphate buffer. Plates were left unsealed and incubated at 25°C in total darkness and either photoinduced at 48 h or injured at 72 h, as described in 2.2.3 and 2.2.4, respectively. Two plates per treatment were inoculated and the entire experiment was repeated once.

2.6.3 Light-induced conidiation of *T. asperellum* on PDA Amended with Glutamine

Light-induced conidiation was investigated in *T. asperellum* LU697 on PDA amended with incremental amounts of glutamine. Conidial suspensions, prepared as described in 2.2.2 were inoculated to potato-dextrose agar (PDA) with 0, 25, 50, 100 or 200 mM L-Glutamine. PDA was prepared according to Appendix 7.2.1. Glutamine was prepared as described in 2.6.1 and added to the agar immediately before pouring. Plates were left unsealed and incubated at 25°C in total darkness and photoinduced at 48 h, as described in 2.2.3. Four plates per treatment were inoculated and the entire experiment was repeated once.

2.6.4 Relationship of Medium pH to Light- and Injury-induced Conidiation in *T. atroviride* on Glutamine-amended Minimal Medium Agar

Changes in the pH values of *T. atroviride* plates at the time of conidiation stimulus were investigated using buffered and unbuffered glutamine-amended MM agar. Buffered and unbuffered glutamine-amended agar was prepared as described in 2.6.1 and 2.6.2. Plates were inoculated with conidial suspensions, as described in 2.2.2, and incubated unsealed at 25°C in total darkness and either photoinduced at 48 h or injured at 72 h, as described in 2.2.3 and 2.2.4, respectively. Two plates per treatment were inoculated and the entire experiment was repeated once. The pH of the agar plates was measured prior to inoculation and at the time of conidiation stimuli, using a Gelplas Double Junction Flat Tip pH probe (BDH [Merck NZ Ltd., Palmerston North, New Zealand]). Light has been reported to induce intracellular acidification in *Trichoderma* (Gresik *et al.*, 1991), which may affect the pH of the medium, therefore readings were taken in the light and in the dark room using safe-red illumination. Values were recorded from under the growing colony and on the uncolonised agar in the photoconidiation experiment and from under the growing colony only in the injury experiment. Measurements were made in triplicate and averaged.

2.6.5 Injury Response of *T. atroviride* and *T. hamatum* at pH 2.8 to 5.6

The effect of pH on injury-induced conidiation was investigated in *T. atroviride* and *T. hamatum* on MM agar with 25 mM glutamine and PDA buffered from pH 2.8 to 5.2 at intervals of 0.4. Buffered MM agar was prepared essentially as follows: 0.2 g $MgSO_4.7H_2O$, 0.9 g K_2HPO_4, 0.2 g KCl, 2 mg $FeSO_4.7H_2O$, 2 mg $ZnSO_4.7H_2O$ and 2 mg $MnCl_2.7H_2O$ were dissolved in 500 mL 2X McIlvaine buffer (citrate/phosphate) (Appendix 7.2.3) and the pH adjusted with HCl or KOH. Water agar was prepared at 2

X concentration to just under the equivalent volume and both were sterilised as described above. Glucose and glutamine were prepared as described in 2.6.1 and combined with the MM components in buffer and water agar immediately prior to pouring. The PDA was prepared in a similar fashion. Potato-dextrose broth (PDB) was dissolved at 2 X concentration in the appropriate McIlvaine buffer and the pH adjusted with HCl or KOH. An equivalent volume of water agar was prepared at 2 X concentration and both were sterilised then combined immediately prior to pouring. Plates were inoculated as described in 2.2.2 then incubated and injured as described in 2.2.4. The pH of the agar plates was measured prior to inoculation and at the time of injury using a Gelplas Double Junction Flat Tip pH probe (BDH [Merck NZ Ltd.]). Measurements were made in triplicate and averaged. Two plates were inoculated per treatment and the entire experiment was repeated twice.

2.6.6 Growth of *T. atroviride* at pH 5.8 to 7.8

The effect of pH 5.8 to 7.8, with intervals of 0.4, on growth of *T. atroviride* was investigated on buffered MM agar with glutamine. Agar was prepared as described in 2.6.1 except Gomori buffers (phosphate/phosphate) (Appendix 7.2.3) were used in place of citrate/phosphate buffers. Plates were inoculated as described in 2.2.2 and incubated for 5 d in total darkness at 25°C. Two plates were inoculated for every pH value and the experiment was repeated once.

2.7 Results

2.7.1 Conidiation Induction on pH-buffered Nitrogen-amended Minimal Medium Agar

The effect of different sources of nitrogen on light- and injury-induced conidiation was examined in five isolates of *Trichoderma* using glutamine and urea as the primary nitrogen sources and KNO_3 as the secondary nitrogen source. No major differences were observed between pH-buffered MM amended with glutamine or urea, however dramatic differences in conidiation and morphology were observed between MM amended with primary nitrogen compared to MM amended with secondary nitrogen.

2.7.1.1 Blue-light Photo-induction of Conidiation

2.7.1.1.1 MM Agar Amended with Primary Nitrogen (pH-buffered)

The influence of primary nitrogen on light-induced conidiation was assessed for all *Trichoderma* isolates using pH-buffered MM agar amended with glutamine or urea. The control plates were incubated in total darkness for 5 d to assess conidiation in the absence of light stimuli. As observed in the PDA experiments (section 2.3.2), conidiation did occur on the control plates for *T. asperellum* and *T. harzianum*, but not for the other isolates. All isolates had a reduced growth rate compared to PDA and the colony morphologies of all isolates were different giving each isolate a characteristic appearance (Figure 2.4A). In *T. hamatum*, there was a thinning of the hyphae at the centre of the plate which extended outwards to a diameter of 3 cm. The colony margins of *T. atroviride* were undulating and the colony appeared to re-grow from the centre outwards on top of the colony. This colony morphology was referred to as a 'stress' morphology. Re-growth from the centre outwards was also observed in *T. asperellum* however the re-growth was less extensive and the margins had a wispy appearance. Sparse conidiation was observed and this was unpigmented to yellow. A spoke-like growth pattern was observed on the surface of the *T. virens* plate. Eight spokes were clearly visible and a yellow pigment was excreted in the centre of the colony extending outwards along the spokes. Multiple spokes were observed in *T. harzianum* and a yellow pigment was observed surrounding a zone of immature conidiation which extended outwards from the centre. The conidiation was reduced in comparison with the PDA experiments where a thick dense mat of mature conidia was produced across the control plates.

Conidiation on pH-buffered MM agar amended with primary nitrogen in response to a single light exposure was observed in *T. asperellum* and *T. harzianum* only, which differed from the PDA experiment where all isolates produced conidia in response to light. At the time of light exposure the colony diameters ranged from 25-30 mm, except for *T. harzianum* which was 10 mm in diameter. The treatment plates of both *T. hamatum* and *T. atroviride* were indistinguishable from controls (Figure 2.4B). A disk of yellow-orange conidia was observed on the *T. asperellum* treatment plates and this correlated with the colony margin at the time of light exposure. This contrasted sharply with the PDA experiments (Figure 2.2C), in which a ring of dark-green pigmented conidia was produced. Plates from one experiment were incubated for a further 5 d and observed again at which time pigmentation was orange to green. Like *T. hamatum* and

T. atroviride, no conidia were observed in *T. virens* in response to light and the yellow pigment appeared to be constrained in a disk which was at the position occupied by the colony margin at the time of light exposure. In addition, the number of spokes appeared to increase relative to the control and were less discernible. In *T. harzianum*, a small disk of concentrated yellow-orange conidia plus some diffuse unpigmented conidia were observed in the centre, which differed from the green pigmented disk observed in Figure 2.2C. Plates from one experiment were incubated further and as with *T. asperellum* pigmentation became orange to green. This disk correlated with the colony margin at light exposure, which was greatly reduced in size compared to Figure 2.2C and to the other isolates on buffered MM with glutamine.

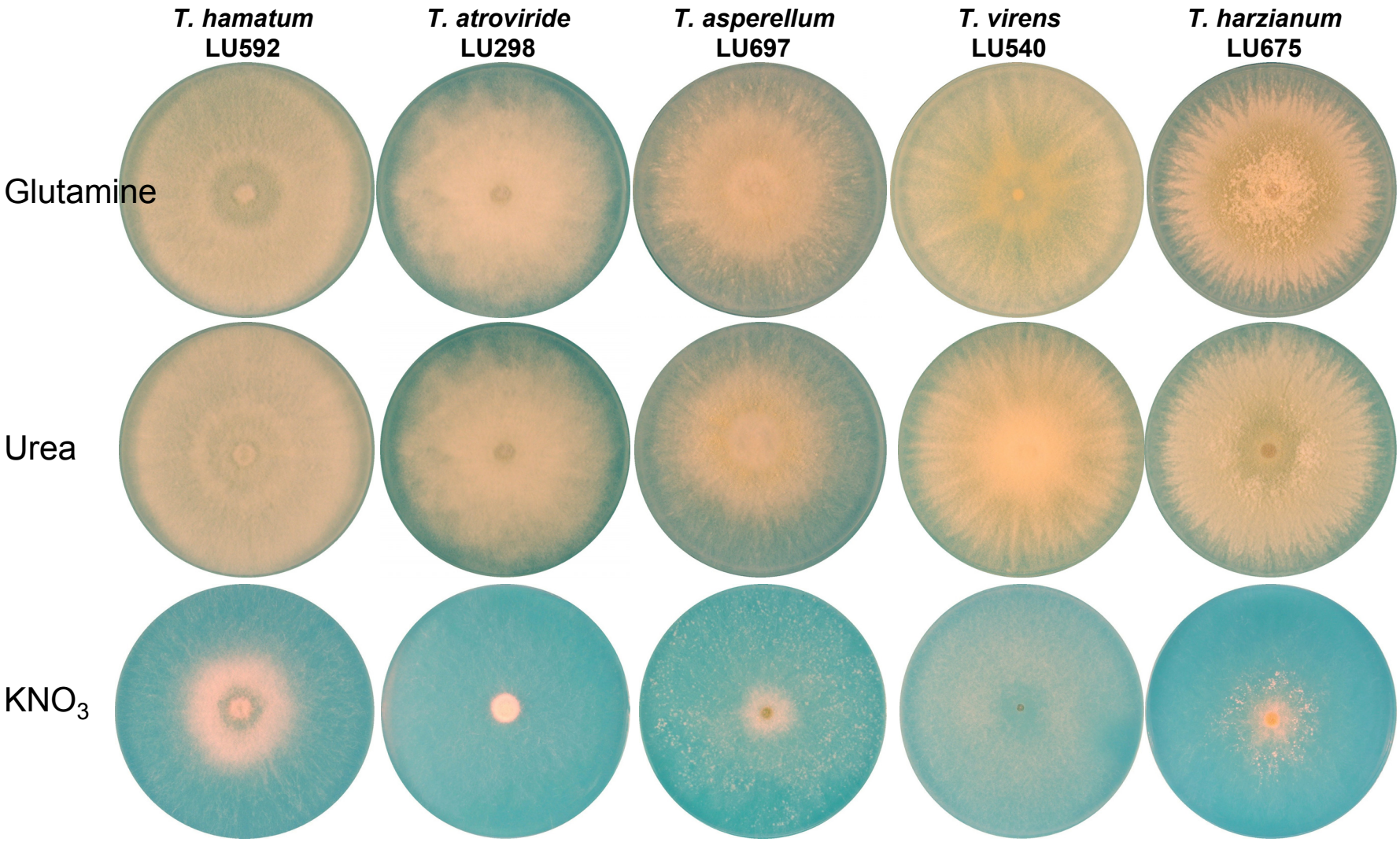


Figure 2.4A. Photoconidiation experiment (control plates) on buffered Minimal Medium amended with glutamine, urea or KNO_3. Plates were grown for 5 d in total darkness.

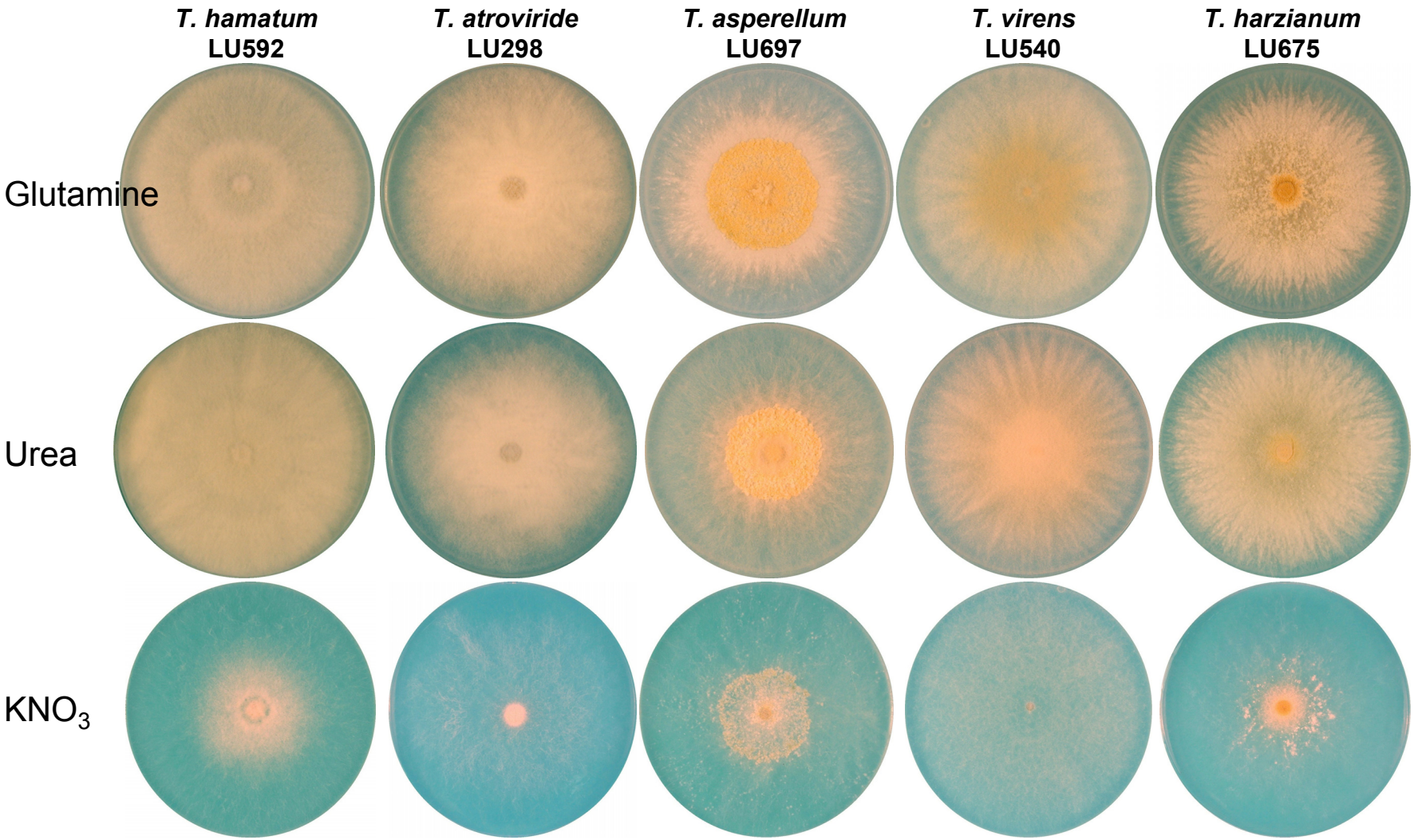


Figure 2.4B. Photoconidiation experiment (treatment plates) on buffered Minimal Medium amended with glutamine, urea or KNO_3. Plates were grown for 48 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

2.7.1.1.2 MM Agar Amended with Secondary Nitrogen (pH-buffered)

The influence of secondary nitrogen on photoconidiation was investigated in all five isolates using pH-buffered MM agar amended with KNO_3. Similar to the primary nitrogen amended MM (1°N + MM) experiments, conidiation did occur on the *T. asperellum* and *T. harzianium* controls. All isolates had a reduced growth rate compared with growth on PDA and slightly reduced growth compared with 1°N + MM. In addition, the colony morphologies were different to those on PDA and 1°N + MM, giving each isolate a characteristic appearance (Figure 2.4A). On the *T. hamatum* plates, mycelial growth was sparse compared with 1°N + MM and the colony appeared to re-grow out from the centre on top of the colony, similar to the stress morphology described for *T. atroviride* in the 1°N + MM experiments. Like *T. hamatum*, growth was sparse in *T. atroviride* compared with the PDA and 1°N + MM experiments. A small area of thickened hyphal growth was observed at the centre of the plate. Growth was also sparse in *T. asperellum* compared with PDA and 1°N + MM. Sparse unpigmented conidiation was observed across the plate and a thickening of the hyphae was observed at the centre. The central inoculum site was pigmented orange-yellow. In *T. virens*, the spoke morphology observed on 1°N + MM was absent on MM amended with KNO_3. Growth was sparse in *T. harzianum* compared with PDA and 1°N + MM. Like *T. asperellum* sparse unpigmented conidia were observed, though this was confined to the centre of the plate surrounding a zone of thickened hyphae. The central inoculum site was pigmented orange-yellow, though more intensely.

Conidiation on pH-buffered MM agar amended with KNO_3 in response to a single light exposure was observed only in *T. asperellum*, which differed from all isolates on PDA and *T. asperellum* and *T. harzianum* on 1°N + MM (Figure 2.4B). A yellow-green conidial ring was produced in *T. asperellum* and this corresponded with the colony margin at the time of light exposure. Sparse conidiation was also observed inside the ring. This morphology differed from the 1°N + MM plates where a yellow-orange disk was formed and PDA where a green ring formed. This isolate was the only one to conidiate in response to light on all of PDA, 1°N + MM and MM amended with KNO_3. On the *T. harzianum* plates a thickening of the hyphae was observed around the central inoculum site, otherwise all other treatment plates were indistinguishable from the controls.

2.7.1.2 Induction of Conidiation by Hyphal Injury

2.7.1.2.1 MM Agar Amended with Primary Nitrogen (pH-buffered)

Injury-induced conidiation on pH-buffered MM agar amended with glutamine or urea was examined in all five isolates. All plates were inoculated at the same time as the photoinduction plates and incubated one day longer as in Section A, therefore the control plates represent 6 d growth compared with 5 d. As described in the photoconidiation experiment, conidiation was only observed on the *T. asperellum* and *T. harzianum* control plates (Figure 2.5A). The *T. hamatum* control plates were indistinguishable from the photoconidiation experiment. In the remaining isolates, the additional 24 h growth was clearly discernible. The yellow-orange pigment observed on the 1°N + MM photoconidiation controls in *T. virens* and *T. harzianum* was more pronounced on the injury controls and in addition a ring of yellow-orange pigment was visible within the spokes towards the edge of the plate in *T. harzianum*.

Conidiation in response to injury was observed in *T. asperellum*, *T. virens* and *T. harzianum* only, which differed from the PDA injury experiment, in which conidiation was observed in all species except *T. hamatum*. No conidiation in response to injury was observed on the treatment plates in both *T. hamatum* and *T. atroviride*, though some thickening of the hyphae was observed along the injury sites in *T. atroviride*, otherwise the culture morphologies were the same as the control plates (Figure 2.5B). Conidiation in response to injury could clearly be seen in *T. asperellum* and conidia were pigmented yellow-orange as observed on the photoconidiation plates, which differed from the PDA experiments where conidia were green. Sparse conidiation was observed in *T. virens* at the site of injury and like *T. asperellum*, this was yellow-orange in colour. In addition to conidia, yellow pigment was produced at the site of stimulus, similar to the photoconidiation plates. Like *T. asperellum* and *T. virens*, an injury response was also observed in *T. harzianum* and this was in addition to the conidiation in the centre observed on the control plates. Conidia were yellow-orange in colour as observed on the 1°N + MM photoconidiation plates, which also differed from PDA where they were green.

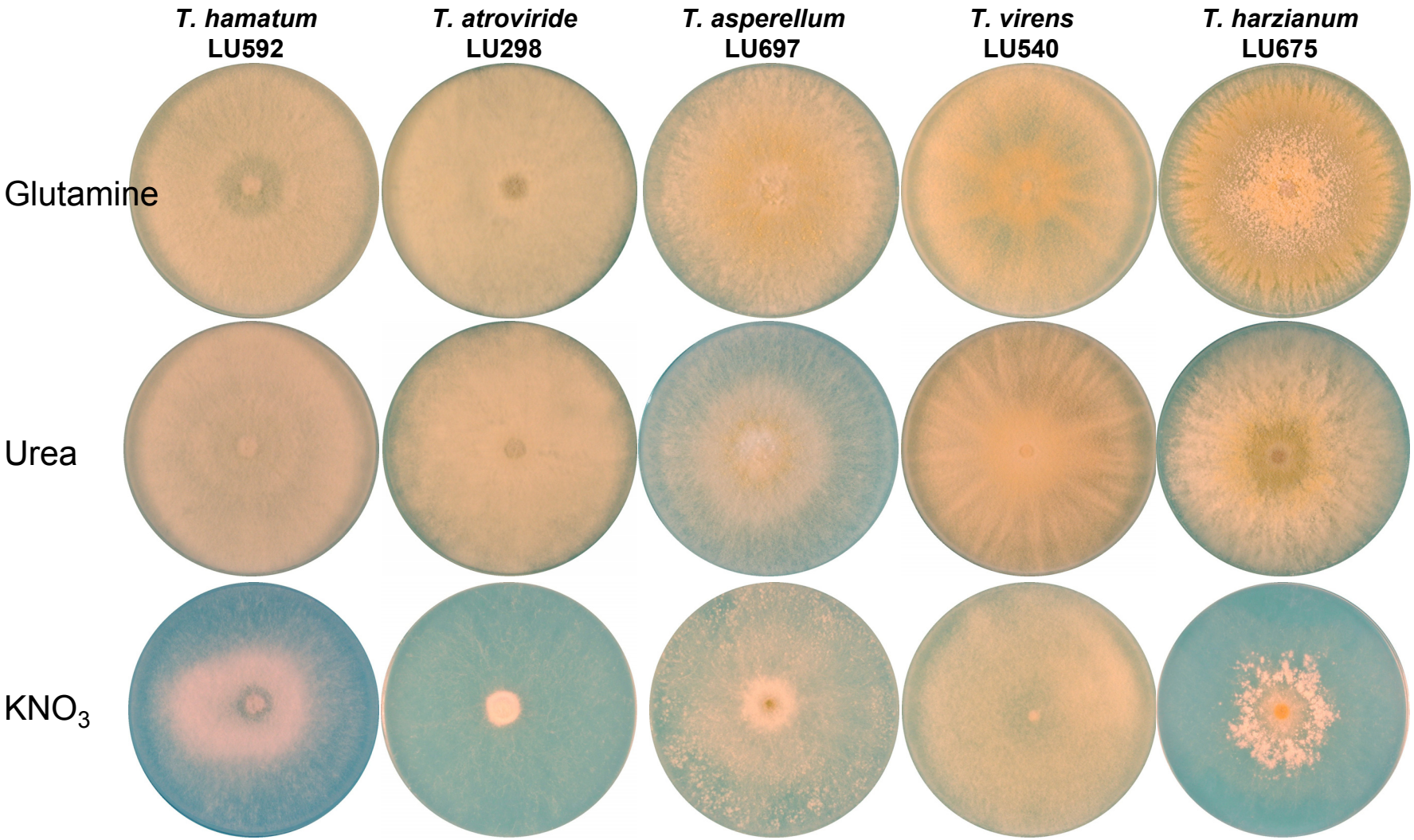


Figure 2.5A. Injury induction experiment (control plates) on buffered Minimal Medium amended with glutamine, urea or KNO_3. Plates were grown for 6 d in total darkness.

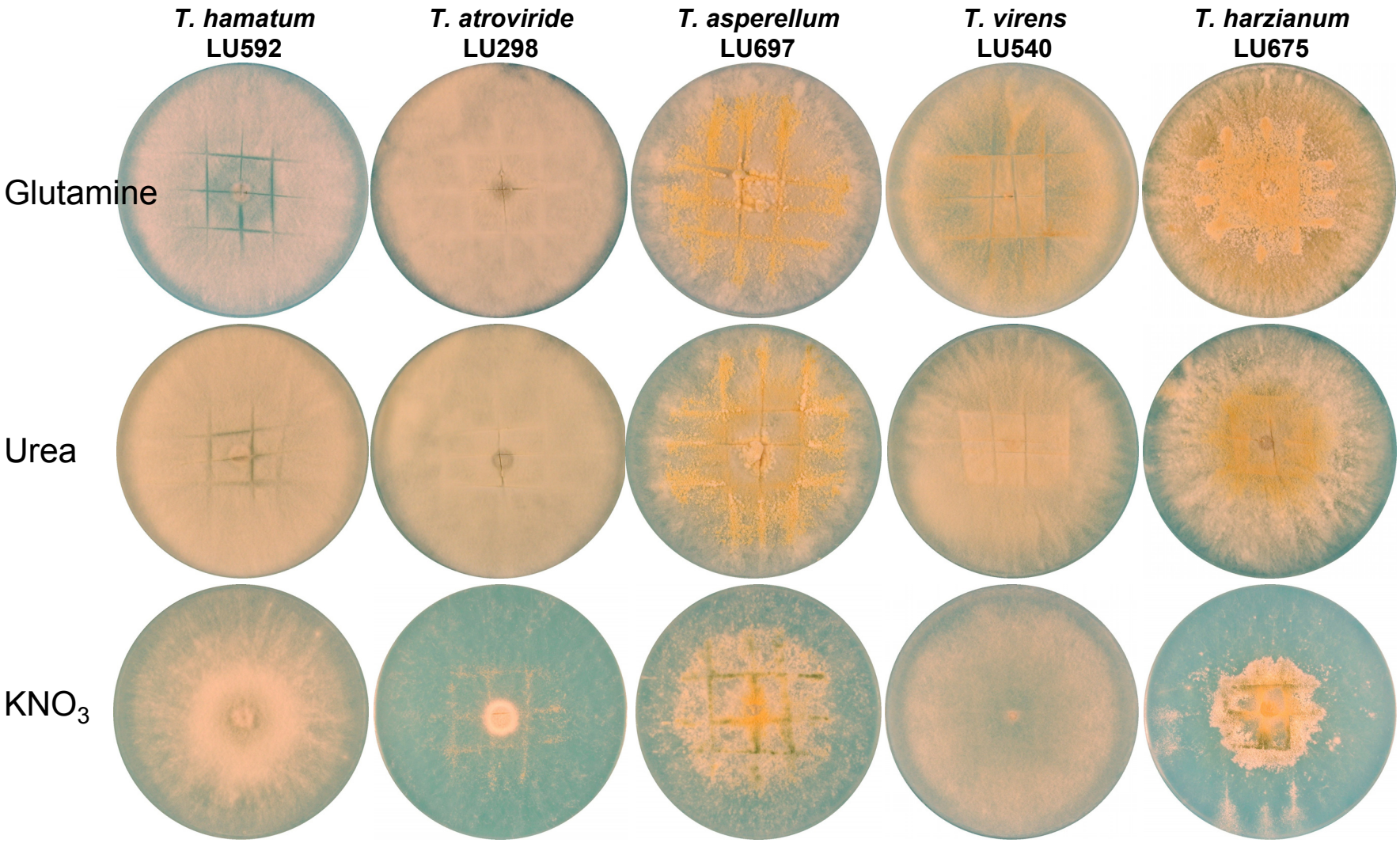


Figure 2.5B. Injury induction experiment (treatment plates) on buffered Minimal Medium amended with glutamine, urea or KNO_3. Plates were grown for 72 h in total darkness, then injury-induced and grown a further 72 h in total darkness.

2.7.1.2.2 MM Agar Amended with Secondary Nitrogen (pH-buffered)

The influence of secondary nitrogen on conidiation induced by injury was investigated in all five isolates on pH-buffered MM agar amended with KNO_3. As described in the KNO_3 photoconidiation experiment and in contrast to the PDA experiment, conidiation was observed on the *T. asperellum* and *T. harzianum* control plates only (Figure 2.5A). In general, growth and conidiation where present, were denser on the control plates compared with the photoconidiation experiment.

Conidiation in response to injury on pH-buffered MM amended with KNO_3 was observed in *T. atroviride*, *T. asperellum* and *T. harzianum*. This differed from PDA where *T. virens* also conidiated and 1°N + MM where only *T. asperellum* and *T. harzianum* conidiated. No difference was discernible between the *T. hamatum* treatment and control plates (Figure 2.5B). Yellow-green pigmented conidia were observed in *T. atroviride*, though conidiation was sparse. This contrasted with 1°N + MM where no conidiation was observed and PDA where they were white to green. Yellow-green conidia were also observed in *T. asperellum* in response to injury and unpigmented conidia were observed between the injury sites. This contrasted with the yellow-orange pigment on 1°N + MM and the green pigment on PDA. Conidiation was also not as intense as on 1°N + MM and PDA. No difference was observed between the control and treatment plates of *T. virens*, which differed from the slight conidial response on 1°N + MM. Like *T. atroviride* and *T. asperellum*, a slight injury response was observed in *T. harzianum*, however this was only within the central area where conidia were also observed on the control plates. In addition, unpigmented spores were observed between the injury sites.

2.7.2 Conidiation Induction on Unbuffered Nitrogen-amended Minimal Medium Agar

The nitrogen assays were pH buffered to ensure that differences in colony phenotype detected for different nitrogen sources were not due to changes in pH of the medium. The experiments were repeated using unbuffered medium which had been acidified to pH 5.4. As observed on the buffered medium, no major differences were observed between unbuffered MM amended with glutamine or urea, however dramatic differences in conidiation and morphology were observed between MM amended with primary nitrogen compared to MM amended with secondary nitrogen.

2.7.2.1 Blue-light Photo-induction of Conidiation

2.7.2.1.1 MM Agar Amended with Primary Nitrogen (Unbuffered)

The contribution of changes in pH to the influence of primary nitrogen on photoconidiation was examined in all isolates using MM agar amended with glutamine or urea, which was unbuffered but acidified to pH 5.4 prior to sterilisation. In contrast to the buffered experiments, conidiation was observed on the *T. asperellum*, *T. virens* and *T. harzianum* control plates, whereas on buffered medium it was observed on *T. asperellum* and *T. harzianum* only. The growth rates appeared higher than on pH-buffered 1°N + MM, though not as fast as on PDA. All isolates displayed distinct colony morphologies different to those observed on pH-buffered 1°N + MM and each isolate had a characteristic appearance (Figure 2.6A). In general, hyphal growth was thicker than observed on the buffered plates and conidiation, where present, was more intense. For control plates, no conidiation was observed in *T. hamatum* or *T. atroviride* and the stress morphology observed on buffered medium in *T. atroviride* was not evident. Yellow-orange pigmented conidia were observed across the *T. asperellum* colony and the colony morphology was different from that on both buffered medium and PDA. The mycelial mat was thick and dense and at the centre of the plate puckering of the agar was observed. Conidiation was also observed in *T. virens* and this contrasted to the buffered medium where no conidia were observed. A central zone of hyphal thickening was observed around the site of inoculation, which was surrounded by a 20 mm wide zone of hyphal thinning, followed by thickened hyphae again. In this outer zone, conidia could be seen on the surface of the plate, and this was covered by dense aerial mycelium, as observed in the PDA experiments. Conidial pigmentation was yellow-green and green at the edge, whereas in the PDA experiments it was green all over. Of all the isolates, *T. harzianum* most resembled the PDA response. Though yellow-green in pigment rather than the green observed in the PDA experiments, profuse conidiation was observed all over the plate in a random fashion. The spoke-like colony morphology observed in the buffered experiments was not observed in any isolate on unbuffered 1°N + MM.

Conidiation on unbuffered MM amended with glutamine or urea in response to a single blue-light exposure was observed in *T. atroviride*, *T. asperellum* and *T. harzianum*. This differed from the buffered experiments in which only *T. asperellum* and *T. harzianum* conidiated and the PDA experiments in which all isolates conidiated. The *T. hamatum* treatment plates were virtually identical to the controls (Figure 2.6B). In *T. atroviride*, a

ring of colourless to yellow pigmented conidia was produced and this correlated with the colony margin at the time of light exposure. A similar response was observed on PDA, however conidia were unpigmented and the ring was not as clearly defined. Similar to the response in the buffered experiments, *T. asperellum* produced a disk of profuse yellow-orange conidia on unbuffered glutamine, which also correlated with the colony margin at the time of exposure. This differed greatly from the PDA experiments where a ring of profuse green pigmented conidia were observed. No difference was observed between the control and treatment plates in *T. virens*, which differed from the pigmentation change on buffered plates and a green ring of conidia in the PDA experiments. In response to light, *T. harzianum* produced a disk of yellow-green conidia and the conidiation occurring randomly across the plate appeared to decrease in intensity. This response is more similar to that observed on PDA than on the buffered glutamine plates.

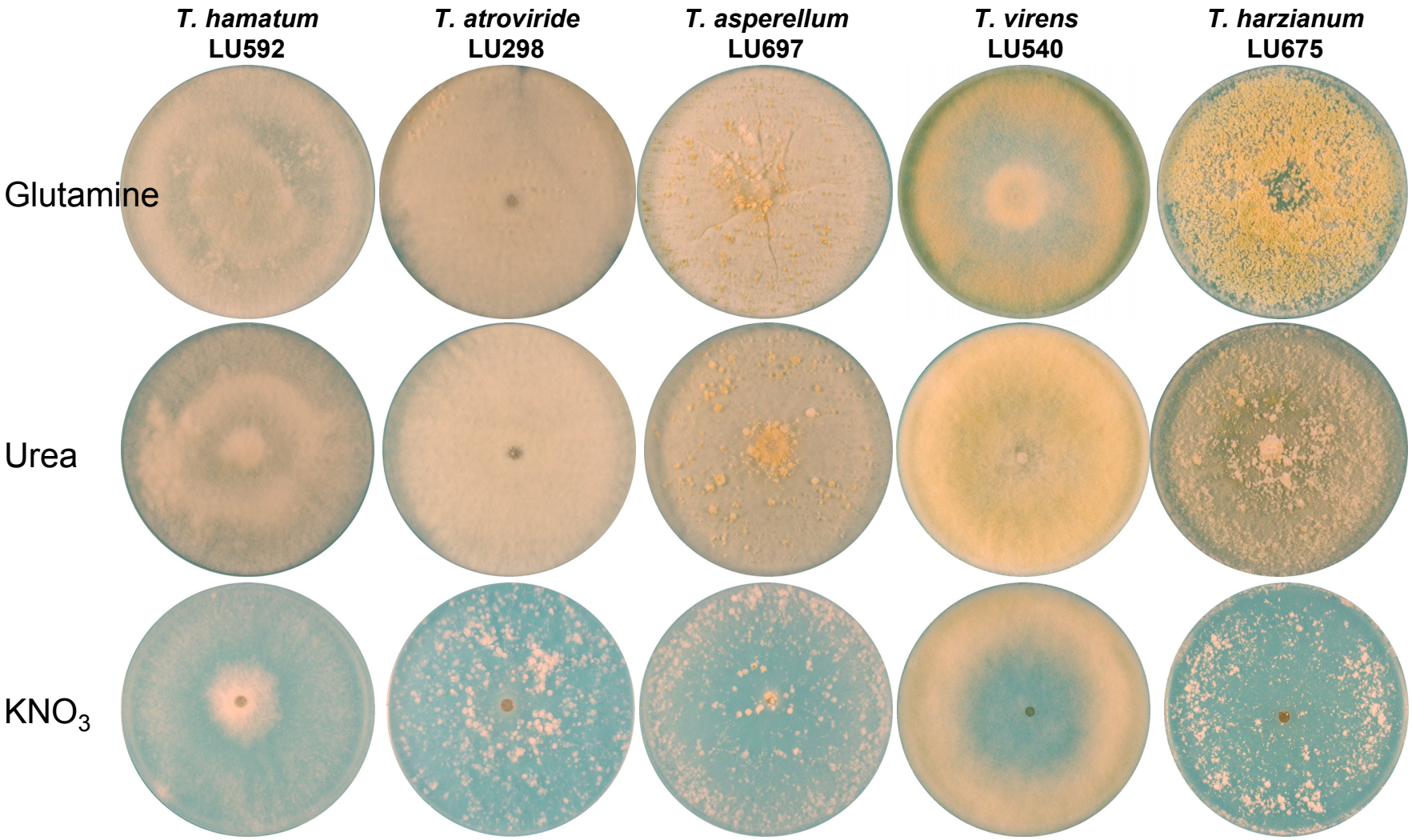


Figure 2.6A. Photoconidiation experiment (control plates) on unbuffered Minimal Medium amended with glutamine, urea or KNO_3. Plates were grown for 5 d in total darkness.

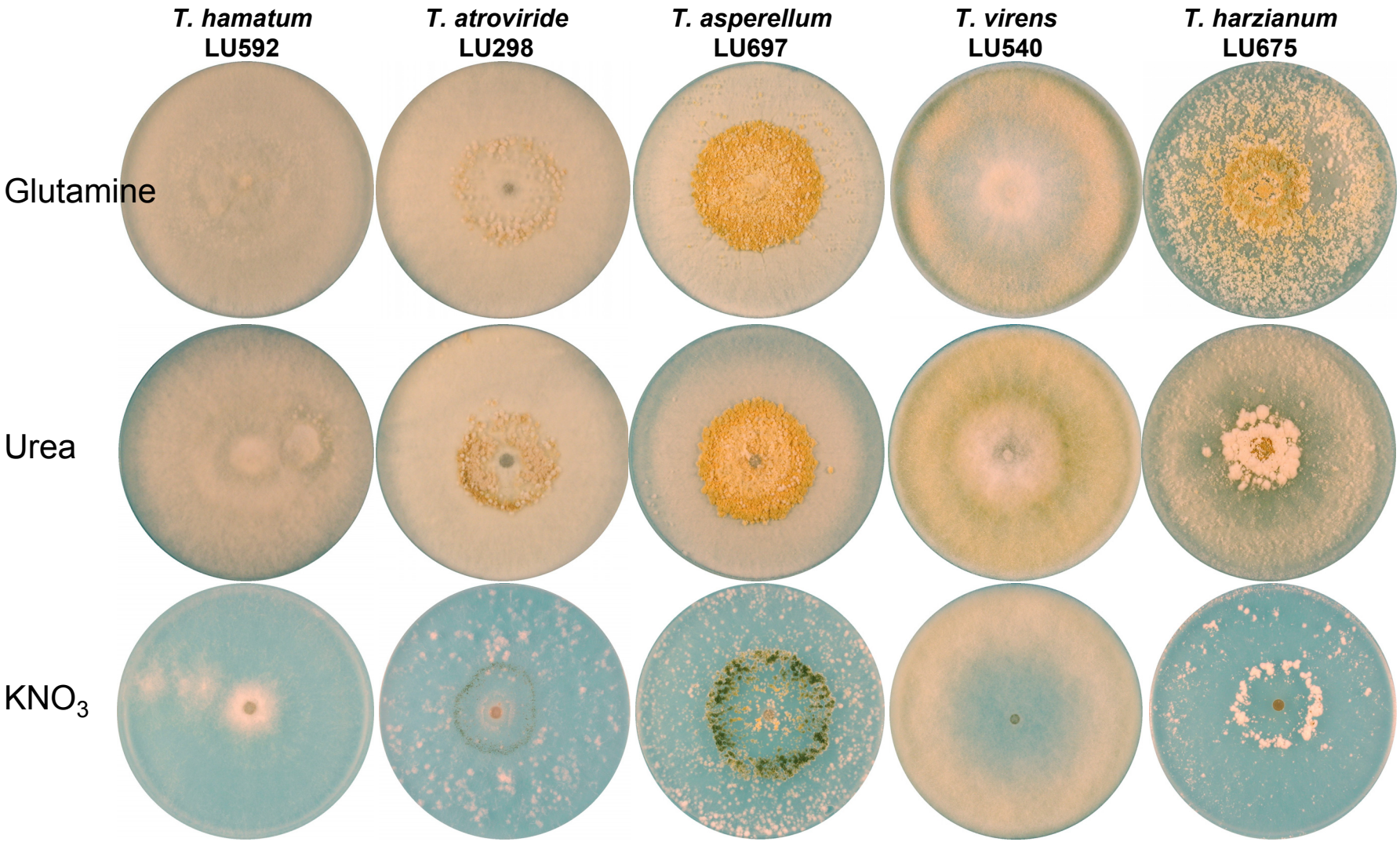


Figure 2.6B. Photoconidiation experiment (treatment plates) on unbuffered Minimal Medium amended with glutamine, urea or KNO_3. Plates were grown for 48 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

2.7.2.1.2 MM Agar Amended with Secondary Nitrogen (Unbuffered)

The contribution of changes in pH to the influence of secondary nitrogen on photoconidiation was examined in all isolates using MM agar amended with KNO_3, which, like the 1°N + MM experiments, was unbuffered but acidified to pH 5.4 prior to sterilisation. Conidiation was observed on the *T. atroviride* control plates in addition to *T. asperellum* and *T. harzianum*, which conidiated in the buffered KNO_3 experiments. In contrast, conidiation was observed on the *T. asperellum*, *T. virens* and *T. harzianum* controls in the PDA experiments. Growth appeared faster compared with the buffered experiment, though not as fast as on PDA. Colony morphologies were different from the buffered KNO_3 and PDA experiments, however the distinction between species on the unbuffered medium was not as discernible and the three conidiating isolates had a similar appearance (Figure 2.6A). Hyphal growth was sparse on the *T. hamatum* and *T. atroviride* control plates. Conidiation was observed across the *T. atroviride* plate and, when incubated for a further 4 d, mature green conidia were produced. A similar morphology was observed in *T. asperellum*, however some yellow-green pigmentation of conidia was apparent. Hyphal growth was considerably more dense on the *T. virens* unbuffered controls compared with the buffered plates and no conidiation occurred. The control plates of *T. harzianum* displayed a similar morphology to that of *T. atroviride* and *T. asperellum*, however conidiation was less dense.

Conidiation on unbuffered MM amended with KNO_3 in response to a single blue-light exposure was observed in *T. atroviride*, *T. asperellum* and *T. harzianum*, which differed from the buffered experiment where it was observed in *T. asperellum* only and on PDA where it was observed in all isolates. Similarly, conidiation also occurred in these isolates on the unbuffered 1°N + MM treatment plates. The *T. hamatum* treatment plates were indistinguishable from the controls (Figure 2.6B). In *T. atroviride*, a clearly defined thin green ring of conidia was observed and this correlated with the position of the colony margin at the time of light exposure. This differed from the ring produced on unbuffered 1°N + MM which was colourless to yellow and on PDA where a diffuse immature partially filled-in ring was produced. A ring of yellow to predominantly green conidia was also produced in *T. asperellum* in response to light and the position of this correlated with the colony margin at the time of light exposure. This response was similar to that observed on the buffered experiments except the conidia were less mature (yellow-green) and the ring was thinner. For *T. virens* no conidiation in response to light was observed and the treatment plates were indistinguishable from the controls. In *T.*

harzianum, a ring of immature conidia were produced which correlated with the position of the colony front at the time of light exposure. This differed strongly from the buffered experiment where there was no conidiation and from the unbuffered 1°N + MM and PDA where disks of conidia rather than rings were produced.

2.7.2.2 Induction of Conidiation by Hyphal Injury

2.7.2.2.1 MM Agar Amended with Primary Nitrogen (Unbuffered)

The contribution of changes in pH to the influence of primary nitrogen on injury-induced conidiation was examined in all isolates using MM agar amended with glutamine or urea, which was unbuffered but acidified to pH 5.4 prior to sterilisation. All plates were inoculated at the same time as the photoinduction plates and incubated one day longer as in Section A, therefore the control plates represent 6 d growth compared with 5 d. There was little difference between the injury induction and photoinduction control plates. The ring of hyphal thinning in *T. hamatum* was much less apparent on the injury plates (Figure 2.7A). There was no discernible difference between the photoinduction and injury-induction controls in the remaining four isolates.

Conidiation in response to injury was discernible in all isolates except *T. hamatum*. This differed from the buffered 1°N + MM experiment in which only *T. asperellum* and *T. harzianum* conidiated, but was the same as observed in the PDA experiment. No response to injury was observed on the *T. hamatum* treatment plates and the culture morphology was the same as the control plates (Figure 2.7B). In *T. atroviride* thickening of the hyphae was observed along the injury sites and a ring of yellow conidia was produced at what was the colony margin at the time of injury stimuli. No ring of conidia was observed on the control plates, therefore the ring was unlikely to be due to accidental light exposure and more likely to be injury-associated. This differed from the buffered experiment where no conidiation occurred and PDA where colourless to green pigmented conidia were observed at the site of injury. In *T. asperellum*, the intensity of conidiation in response to injury was significantly greater than observed in the buffered experiments. Conidia were produced in between as well as along the injury sites, which made it difficult to discern where injury occurred. Conidia were also yellow-orange, unlike the buffered plates where mature green conidia were observed at the position of the colony margin when injured. This response differed from the response on PDA where conidia were green in pigment and the injury sites were clearly defined. Conidiation in response to injury was also observed in *T. virens*, however

conidiation was more intense and mature in pigmentation at the outer edge of the colony compared to the buffered 1°N + MM and PDA experiments. Conidia were pigmented orange to green. In response to injury, a thick mat of conidiation was produced on the *T. harzianum* treatment plates and this was significantly more intense than on the buffered plates. Conidiation occurred in between and all along the injury sites as observed in *T. asperellum*, though much denser so that the injury sites were barely discernible. Conidia were yellow-orange and green at the position of the colony margin when injured, which was also observed in *T. asperellum*. This differed from the PDA experiment, in which conidia were green and the injury sites could be discerned.

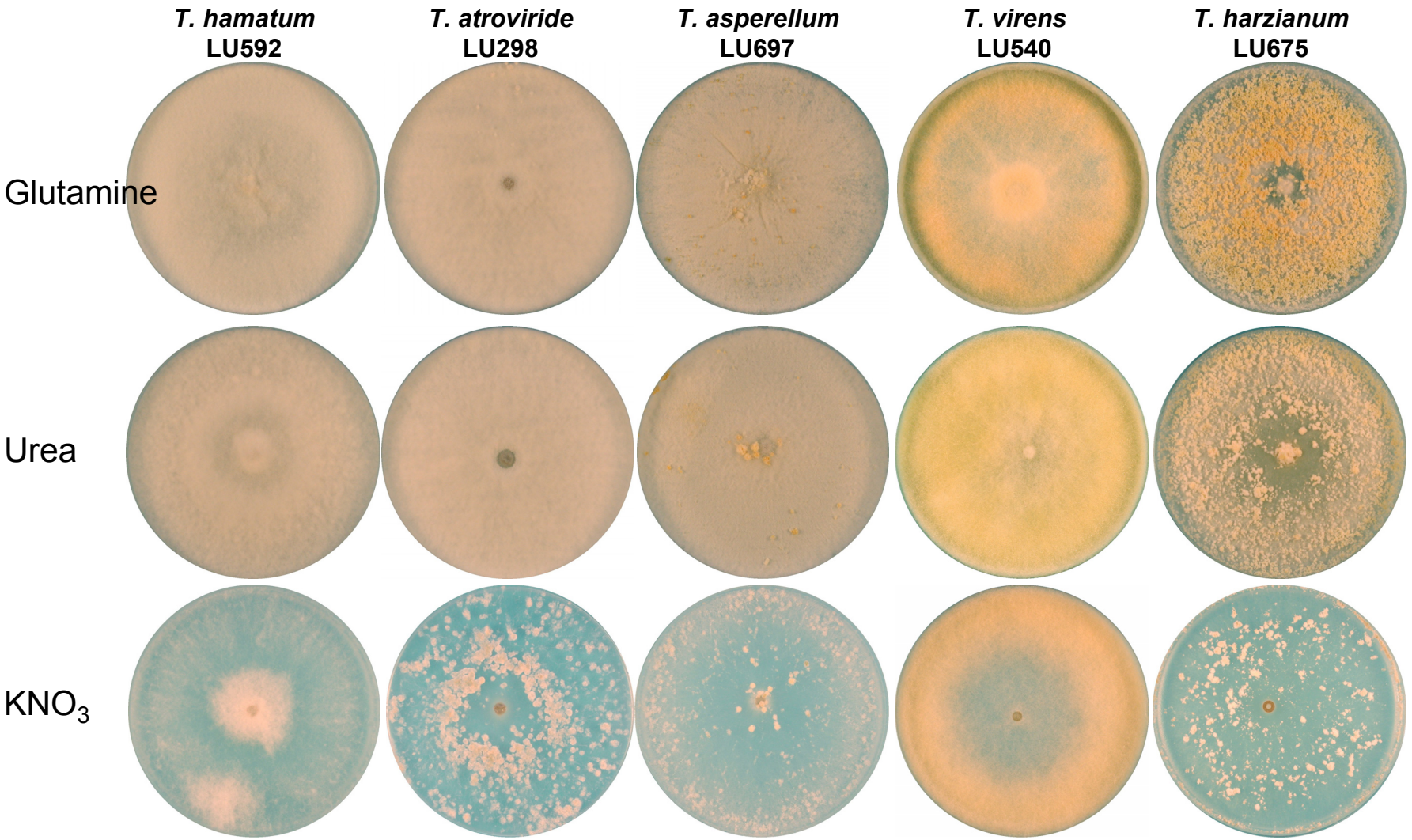


Figure 2.7A. Injury induction experiment (control plates) on unbuffered Minimal Medium amended with glutamine, urea or KNO_3. Plates were grown for 6 d in total darkness.

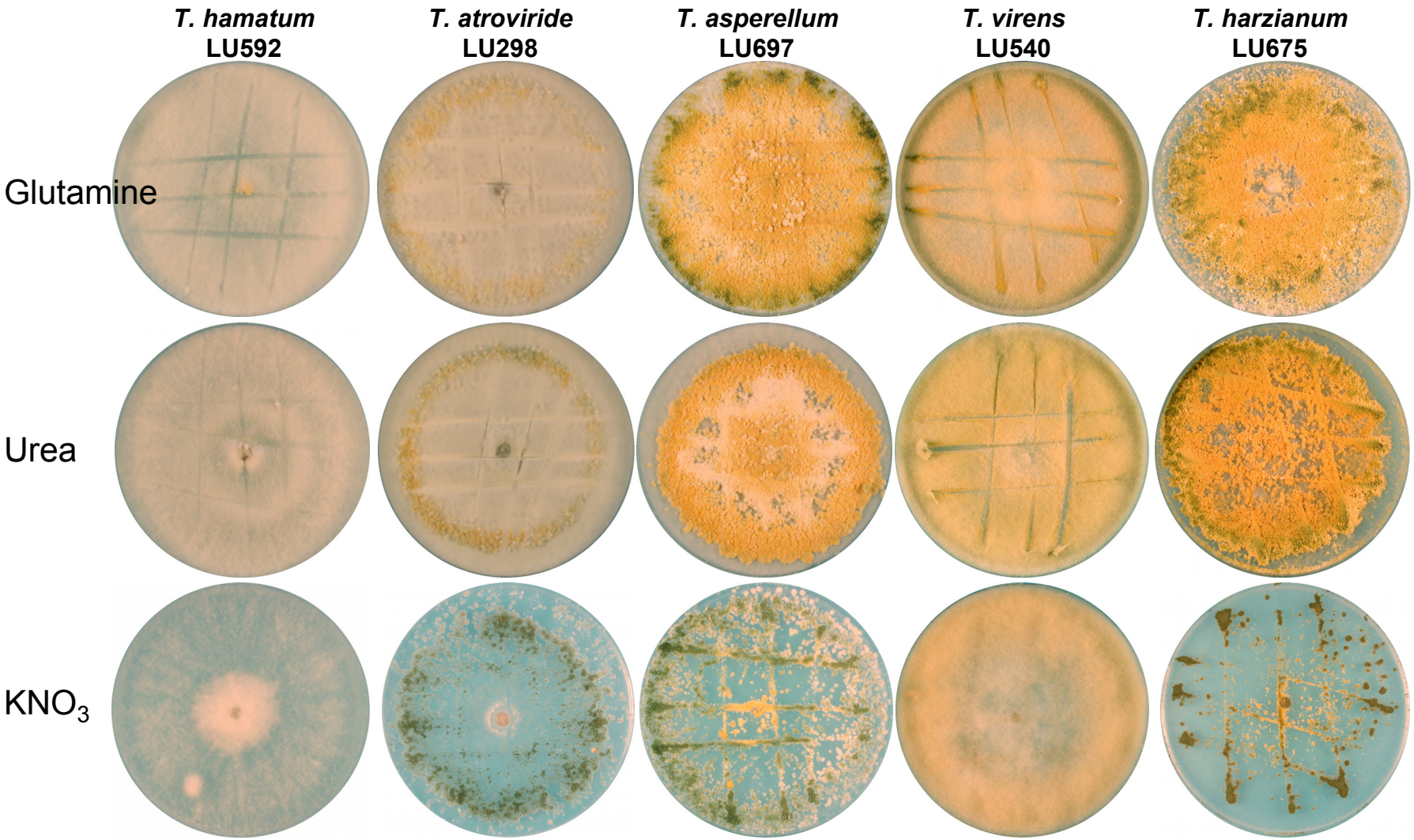


Figure 2.7B. Injury induction experiment (treatment plates) on unbuffered Minimal Medium amended with glutamine, urea or KNO_3. Plates were grown for 72 h in total darkness, then injury-induced and grown a further 72 h in total darkness.

2.7.2.2.2 MM Agar Amended with Secondary Nitrogen (Unbuffered)

The contribution of changes in pH to the influence of secondary nitrogen on injury-induced conidiation was examined in all isolates using MM agar amended with KNO_3, which was unbuffered but acidified to pH 5.4 prior to sterilisation. As described previously, the injury control plates represented 6 d growth compared to 5 d growth on the photoconidiation controls. As observed on unbuffered 1°N + MM, in most isolates there was little difference between the unbuffered KNO_3 injury and photoconidiation control plates. Other than a slight thickening of the mycelium, there was no significant difference between the *T. hamatum* injury and photoconidiation controls (Figure 2.7A). In *T. atroviride*, there was some clustering of conidia in a ring 20 mm from the centre. There was no discernible difference between the *T. asperellum* injury and photoconidiation control plates. In *T. virens*, the hyphal growth was thicker in comparison with the photoconidiation controls and some conidiation was observed at the edge of the plate. In *T. harzianum*, the level of conidiation appeared to increase compared with the photoconidiation controls plates.

Conidiation in response to injury on unbuffered KNO_3 was observed in *T. atroviride*, *T. asperellum* and *T. harzianum* as described in the buffered KNO_3 experiments, however the response was much stronger. No conidiation in response to injury was observed in *T. hamatum* and there was no discernible difference between the control and treatment plates (Figure 2.7B). Green pigmented conidia were produced in response to injury in *T. atroviride*, however only on the outer portion of the colony giving the response a ring-like appearance. This is in contrast to the immature conidia which were produced evenly along the injury sites in the buffered experiment and the colourless to green conidia observed on PDA. A similar response was observed in the glutamine unbuffered experiment in which a ring of yellow conidia was produced. A much stronger conidiation response was observed on the *T. asperellum* treatment plates compared with the buffered experiment. Conidia were observed along the injury sites and, at the position of the colony margin at the time of injury, conidia were observed between the injury sites giving a ring-like appearance. Conidia were yellow-green at the centre of the colony and green in the outer portion, whereas in the buffered experiment conidia were more mature in the central portion of the colony. This differed from the response on PDA where profuse green conidiation was produced evenly along the injury sites. In *T. virens* the zone of hyphal thinning observed in the controls was absent and a thickening of the hyphae along the injury site was observed. The injury response in *T. harzianum*

was similar to that in *T. atroviride*; conidiation was more intense than on buffered medium and pigmentation was yellow-green in the centre and green in the outer portion of the colony. In contrast, conidia were yellow-green on the buffered medium and considerable conidiation was apparent between the injury sites. Considerable conidiation between the injury sites was also observed on PDA, however conidia were green in pigment.

Results from the nitrogen-amended MM agar experiments are summarised in Table 2.2 below.

Table 2.2. Summary of conidiation in response to primary or secondary nitrogen. X = no conidial response to stimuli observed. Ring = photoconidiation ring. Disk = Photoconidiation was disk-like. Conidia = conidiation in response to injury.

		T. hamatum		***T. atroviride***		***T. asperellum***		***T. virens***		***T. harzianum***	
	N	**BUFF**	**UB**	**BUFF**	**UB**	**BUFF**	**UB**	**BUFF**	**UB**	**BUFF**	**UB**
Light	1°	X	X	X	ring	disk	disk	X	X	disk	disk
	2°	X	X	X	ring	ring	ring	X	X	X	ring
Injury	1°	X	X	X	conidia	conidia	conidia	conidia	conidia	conidia	conidia
	2°	X	X	conidia	conidia	conidia	conidia	X	X	conidia	conidia

2.7.3 Light-induced Conidiation of *T. asperellum* on PDA Amended with Glutamine

The production of a disk of conidia in response to light from *T. asperellum* colonies grown on buffered MM with glutamine suggested that glutamine promoted conidiation across the colony. To test this hypothesis, photoconidiation was investigated on PDA amended with incrementally increasing amounts of glutamine. As observed in Section A (Figure 2.2C), *T. asperellum* produced a ring of conidia in response to light on PDA with no amendment and the location of the ring correlated with the colony margin at the time of light exposure (Figure 2.8). As the level of glutamine increased the ring became thicker and conidia were observed inside the ring, eventually forming a disk of conidia at 100 mM glutamine. In addition, the growth rate reduced as the level of glutamine rose and this was most evident at 200 mM.

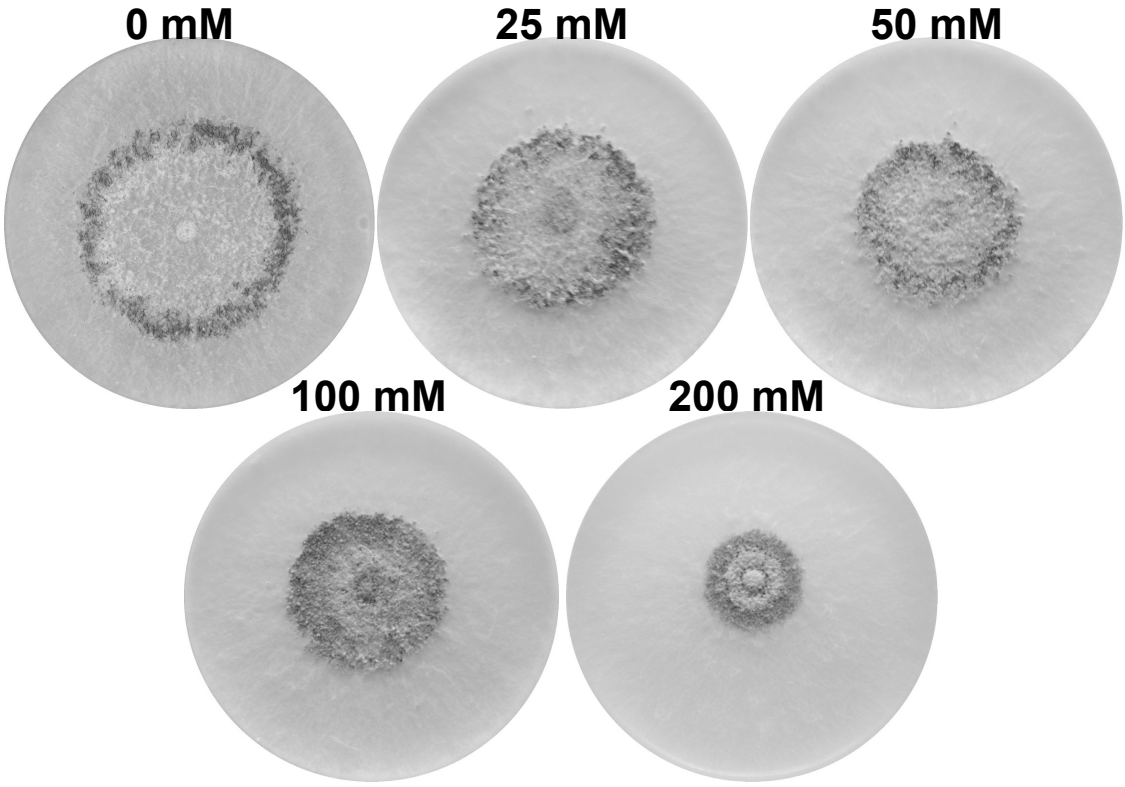


Figure 2.8. Light-induced conidiation in *T. asperellum* on PDA amended with L-Glutamine. Glutamine concentrations are indicated above each plate.

2.7.4 Relationship of Medium pH to Light- and Injury-induced Conidiation in *T. atroviride* on Glutamine-amended Minimal Media Agar

Differences in conidiation between the buffered and unbuffered medium in 2.7.1 and 2.7.2 may have been due to changes in pH, which suggested the observed conidiation responses may be pH associated. The experiment was repeated with *T. atroviride* on buffered and unbuffered glutamine-amended MM agar and the pH values of the agar under the growing colonies were measured at the time of conidiation stimuli, that is 48 h for the photoconidiation plates and 72 h for the injury plates. Whilst the pH range of the buffered medium remained within the range 5.2 – 5.6 at both light and injury stimuli, the pH varied greatly on the unbuffered plates (Figure 2.9). Conidiation in response to stimuli coincided with the lowest pH values. This was particularly evident on the injury treatment plates where conidiation in response to injury occurred only where the medium was at pH 4.8 rather than across the whole colony. There was no difference between pH readings taken in the light or in the dark room.

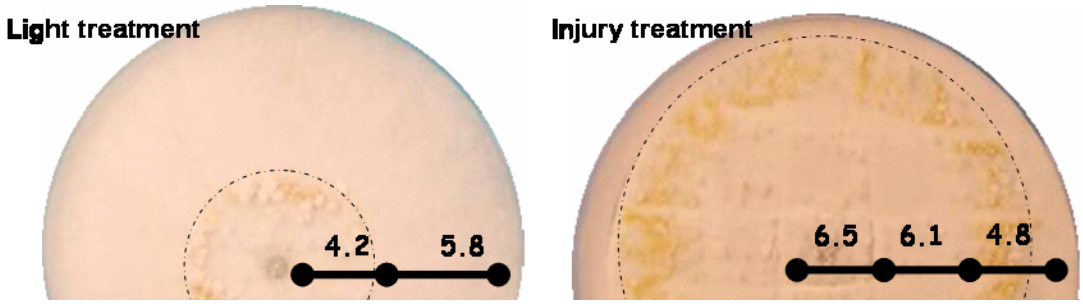


Figure 2.9. *Trichoderma atroviride* conidiation in response to stimuli on unbuffered MM with glutamine. The pH values of the agar under the colony at the time of stimuli are indicated. The dashed lines represent the colony margins at the time of treatment.

2.7.5 Injury Response of *T. atroviride* and *T. hamatum* at pH 2.8 to 5.6

The previous experiments (2.7.1, 2.7.2 and 2.7.4) suggested a relationship exists between ambient pH and conidiation response to stimuli when *Trichoderma* cultures are grown on MM agar amended with either primary or secondary nitrogen. In *T. atroviride*, conidiation in response to stimuli coincided with a region of low ambient pH at the colony margin, suggesting this to be a requirement for conidiation. It was hypothesised that if *T. atroviride* was grown at a low ambient pH then it should conidiate in response to injury and this response should occur evenly along the injury site. To test this hypothesis, *T. atroviride* LU298 and *T. hamatum* LU592 were grown on glutamine-amended MM agar and PDA, which was citrate/phosphate buffered from 2.8 to 5.6, and subjected to mycelial injury as previously described. Previously *T. hamatum* had shown no conidial injury response on nitrogen-amended MM agar, however sparse conidiation between the injury sites had been observed on PDA (Figure 2.3B). This response resembled the injury response for *T. atroviride* on unbuffered glutamine-amended MM agar, therefore *T. hamatum* was included in the experiment also. All media were buffered to ensure the pH of the agar was consistent across the colony and the pH of the agar was measured throughout the experiment.

2.7.5.1 *Trichoderma atroviride*

On glutamine-amended MM agar, buffered from pH 2.8 to 5.6, conidial response to injury in a pH dependent manner was observed, though conidiation was sparse and did not occur evenly along the injury sites. No conidiation was observed on the 6 d old control plates (Figure 2.10A). At pH 5.2 and 5.6 the stress morphology described in 2.7.1.1.1 was observed and this was more apparent at pH 5.6. A thickening of the

hyphae around the injury site was observed on all treatment plates and at pH 2.8 and 3.2 only sparse conidiation was observed at the outer edges of the colony and these appeared to correlate with injury sites (2.10B).

On PDA, conidial response to injury in a pH-dependent manner was clearly observed and as described on 1°N + MM this occurred at the lowest pH. Abundant immature conidiation was observed in the centre of the pH 2.8 control plate and this rapidly decreased to a couple of white tufts at pH 4.0 (Figure 2.11A). No conidiation was observed at the higher pH values. From pH 2.8 to 4.4 only a zone of hyphal thinning was observed at the centre of the colony and at pH 5.6 the colony resembled the previously described stress morphology. Conidiation in response to injury across the whole colony was clearly observed on the pH 2.8 treatment plates (Figure 2.11B). Dense thickened hyphae occurred along the injury site and from within this colourless to green pigmented conidia were observed. The intense background conidiation described on the pH 2.8 control plate could be easily discerned within the grid pattern of the injury sites. Sparse conidiation was observed at the injury sites on the pH 3.2 plates and the hyphal thickening rapidly decreased in intensity from pH 3.2 to 4.0. The pH values of both the MM agar and PDA plates did not alter significantly throughout the experiment.

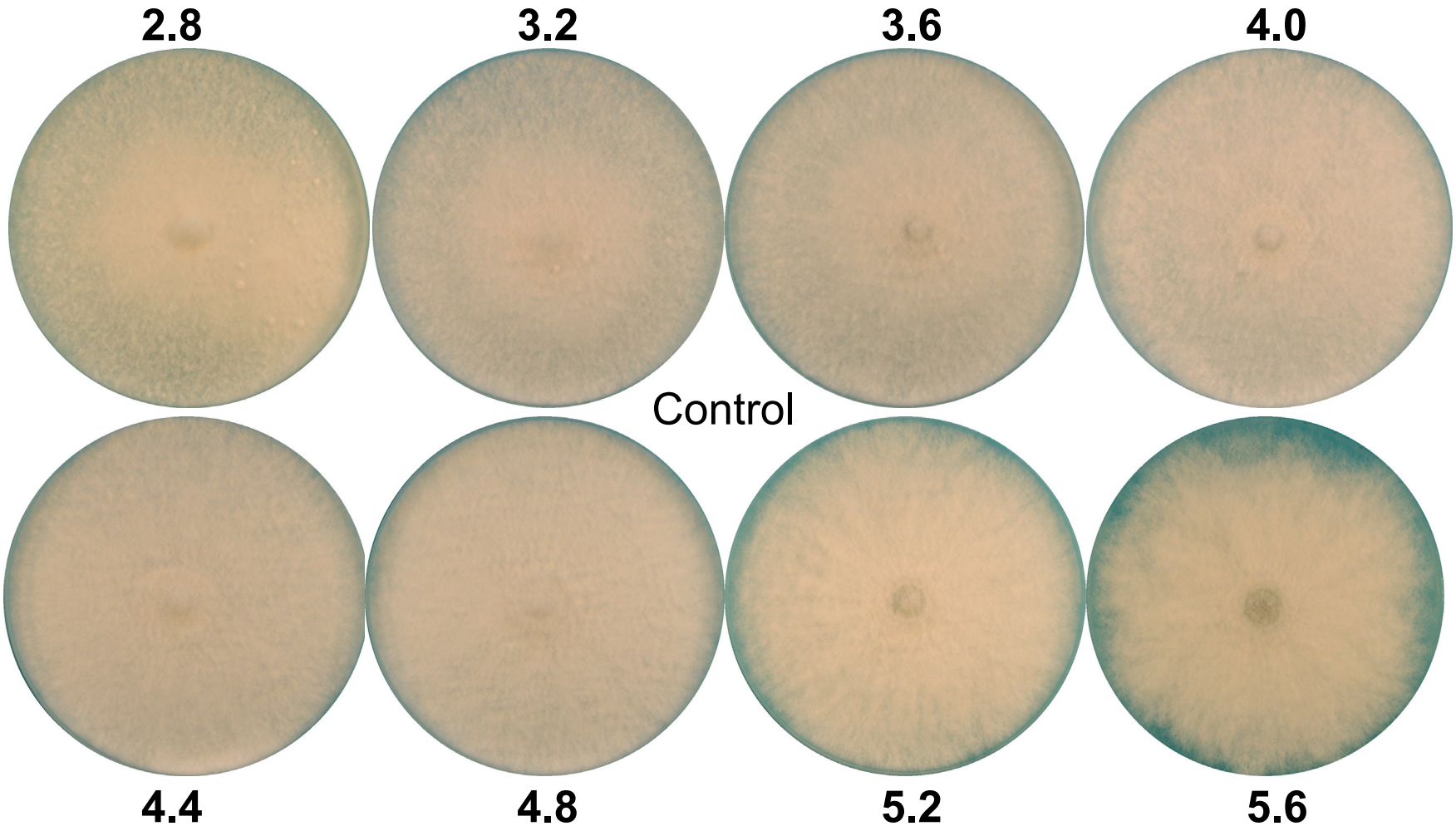


Figure 2.10A. *Trichoderma atroviride* injury induction experiment (control plates) on citrate/phosphate buffered (2.8 to 5.2) MM agar amended with glutamine. Cultures were grown for 6 d in total darkness.

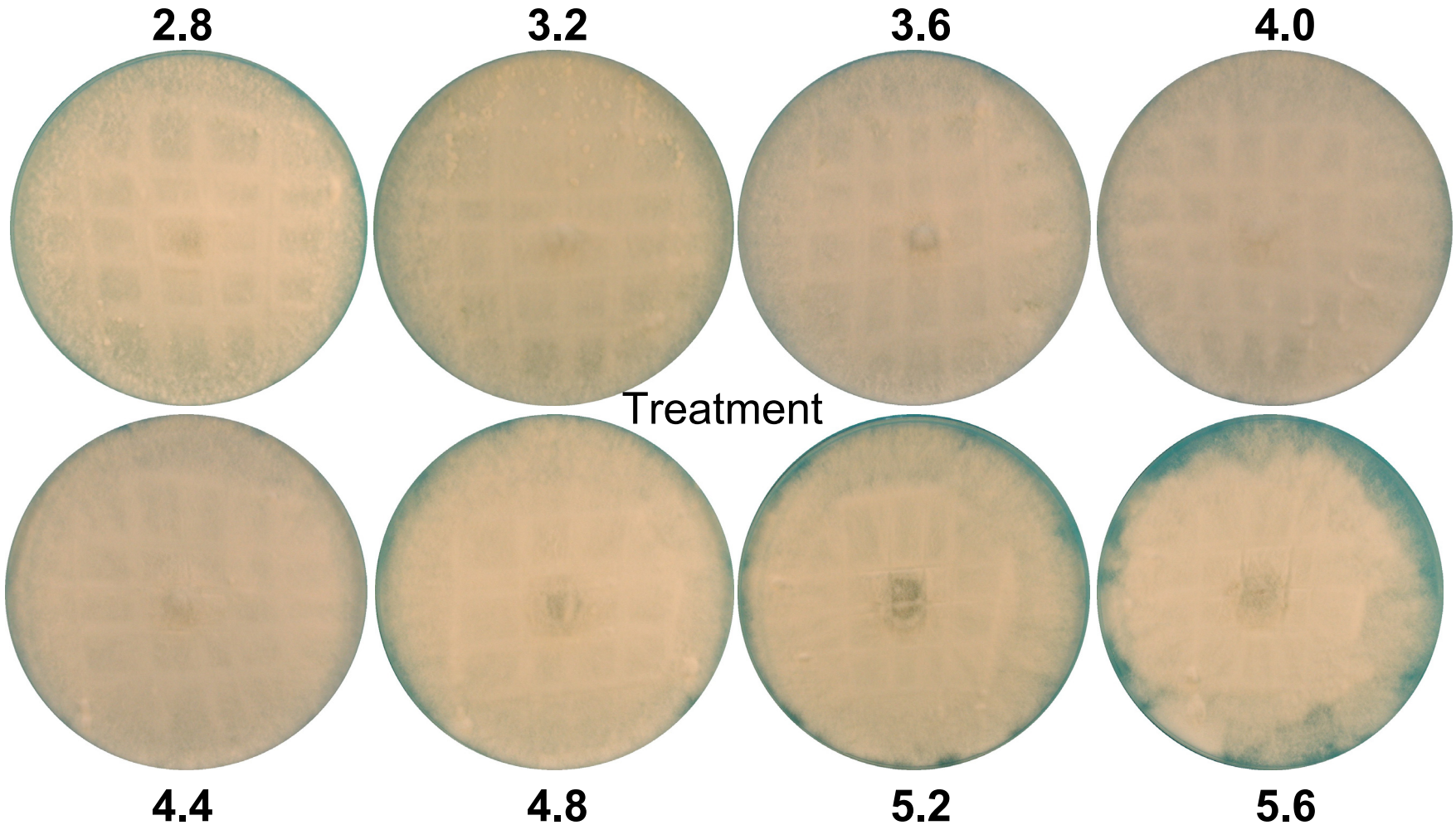


Figure 2.10B. *Trichoderma atroviride* injury induction experiment (treatment plates) on citrate/phosphate buffered (2.8 to 5.2) MM agar amended with glutamine. Cultures were grown for 6 d in total darkness. Plates were grown for 72 h in total darkness, then injury-induced and grown a further 72 h in total darkness.

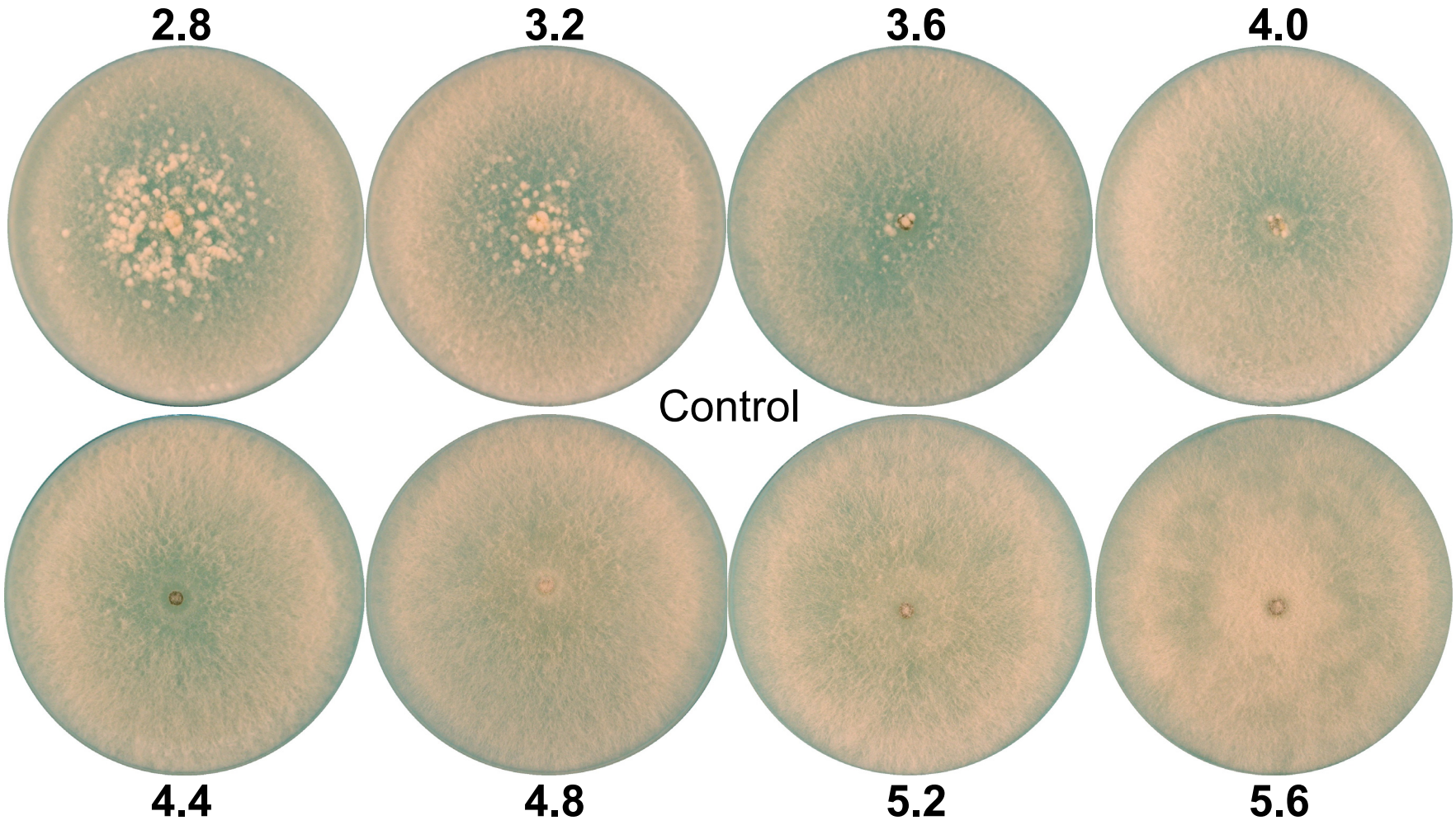


Figure 2.11A. *Trichoderma atroviride* injury induction experiment (control plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Cultures were grown for 6 d in total darkness.

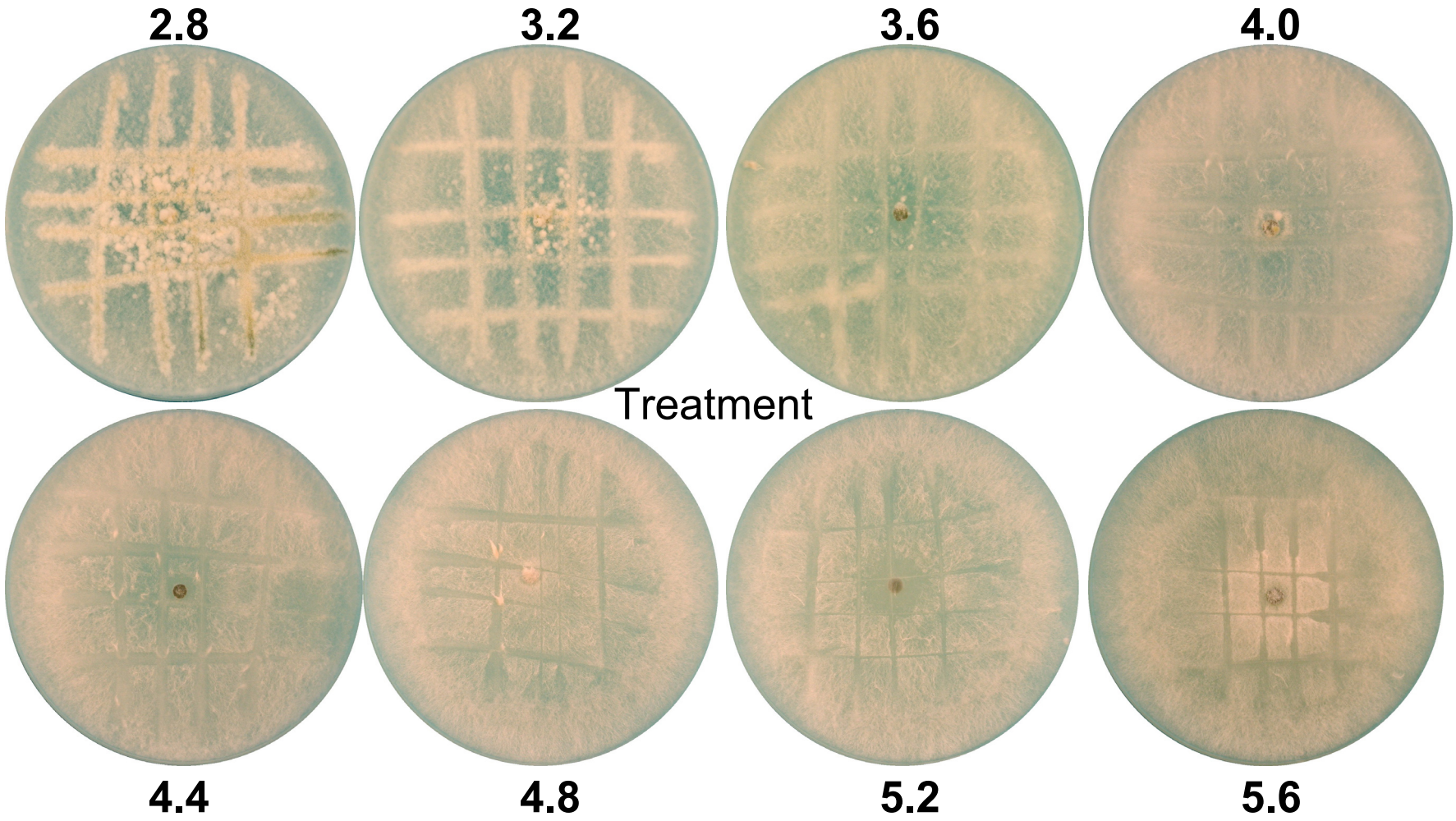


Figure 2.11B. *Trichoderma atroviride* injury induction experiment (treatment plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Cultures were grown for 6 d in total darkness. Plates were grown for 72 h in total darkness, then injury-induced and grown a further 72 h in total darkness.

2.7.5.2 *Trichoderma hamatum*

The influence of pH on injury induced conidiation was also assessed for *T. hamatum* on agar medium buffered from 2.8 to 5.6. No conidiation was observed on either the control or treatment glutamine-amended MM agar plates at any pH (Figure 2.12A & B). Hyphal growth was dense on all control plates and the zone of hyphal thinning observed in 2.7.1.1 was readily apparent at pH 5.6 and became less apparent as the pH lowered. On the treatment plates no response to injury was observed and plates resembled their respective controls (Figure 2.12B). In contrast, conidiation in response to injury in a pH-dependent manner was observed in *T. hamatum* when grown on PDA. On the 6 d old control plates sparse immature conidiation was observed in the centre of the colony at pH 2.8 and 3.2 only and this was more intense at pH 3.2 (Figure 2.13A). A 70 mm diameter zone of hyphal thinning was observed at pH 2.8 and this decreased in size as the pH rose. On the treatment plates immature to yellow-green conidiation along the injury sites was observed at pH 2.8 to 3.6 and this was more intense at pH 2.8 (Figure 2.13B). The conidiation was concentrated at the centre of the colony and was not evenly dispersed along the injury site as was observed on the *T. atroviride* PDA plates. The pH values of both the MM agar and PDA plates did not alter significantly throughout the experiment. This was the first time in these studies that a clear injury response had been observed in *T. hamatum*.

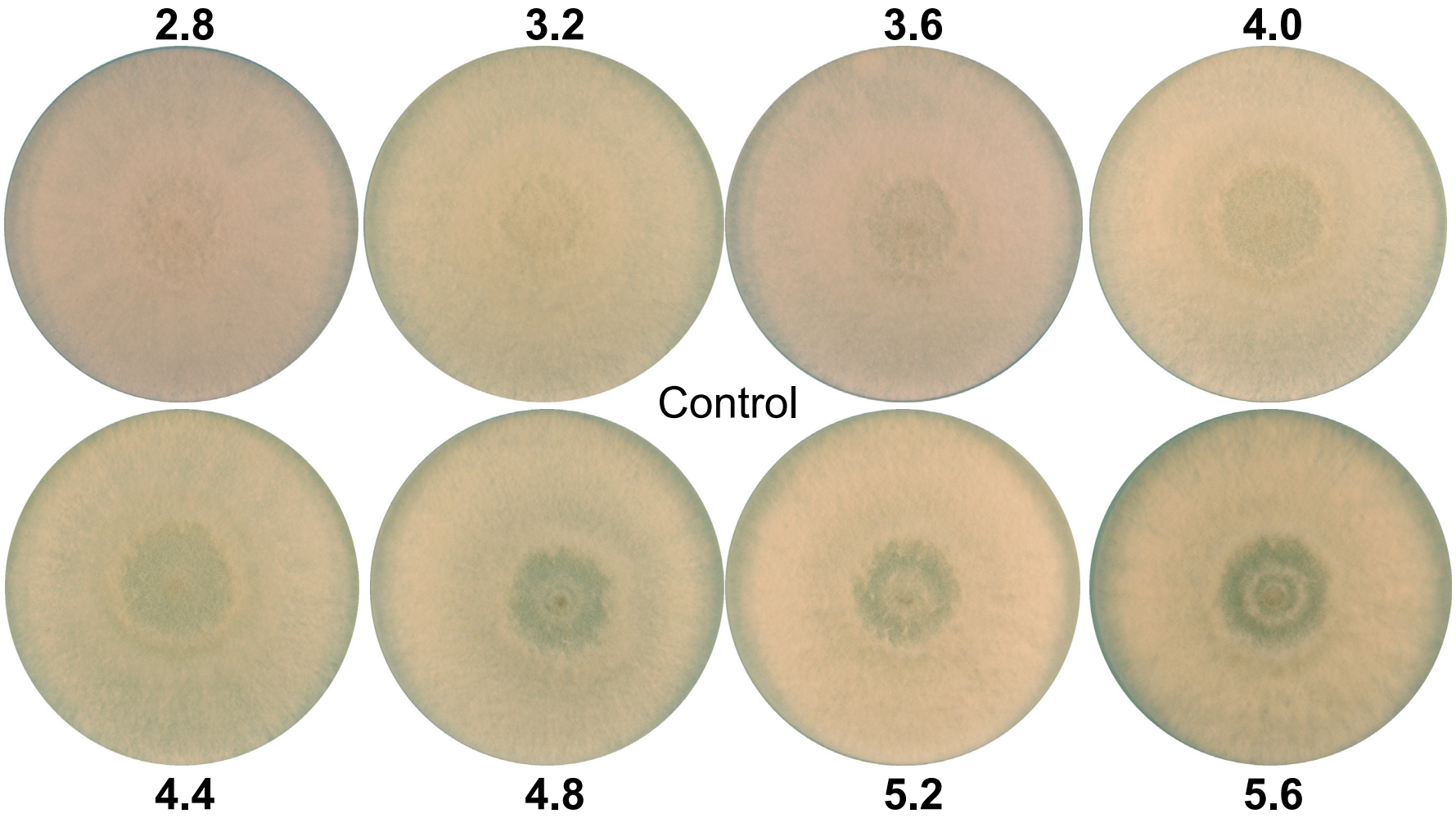


Figure 2.12A. *Trichoderma hamatum* injury induction experiment (control plates) on citrate/phosphate buffered (2.8 to 5.2) MM agar amended with glutamine. Cultures were grown for 6 d in total darkness.

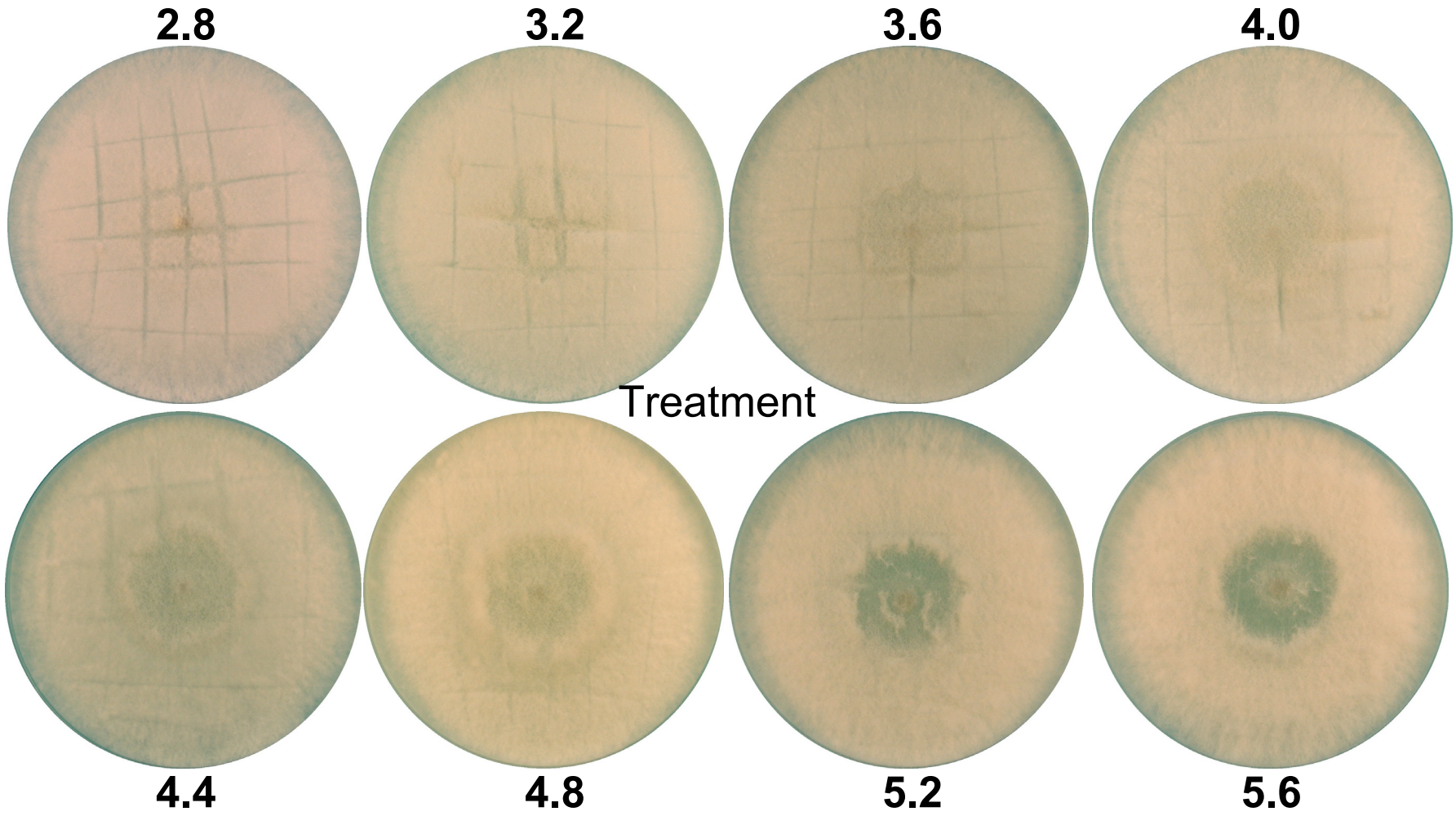


Figure 2.12B. *Trichoderma hamatum* injury induction experiment (treatment plates) on citrate/phosphate buffered (2.8 to 5.2) MM agar amended with glutamine. Cultures were grown for 6 d in total darkness. Plates were grown for 72 h in total darkness, then injury-induced and grown a further 72 h in total darkness.

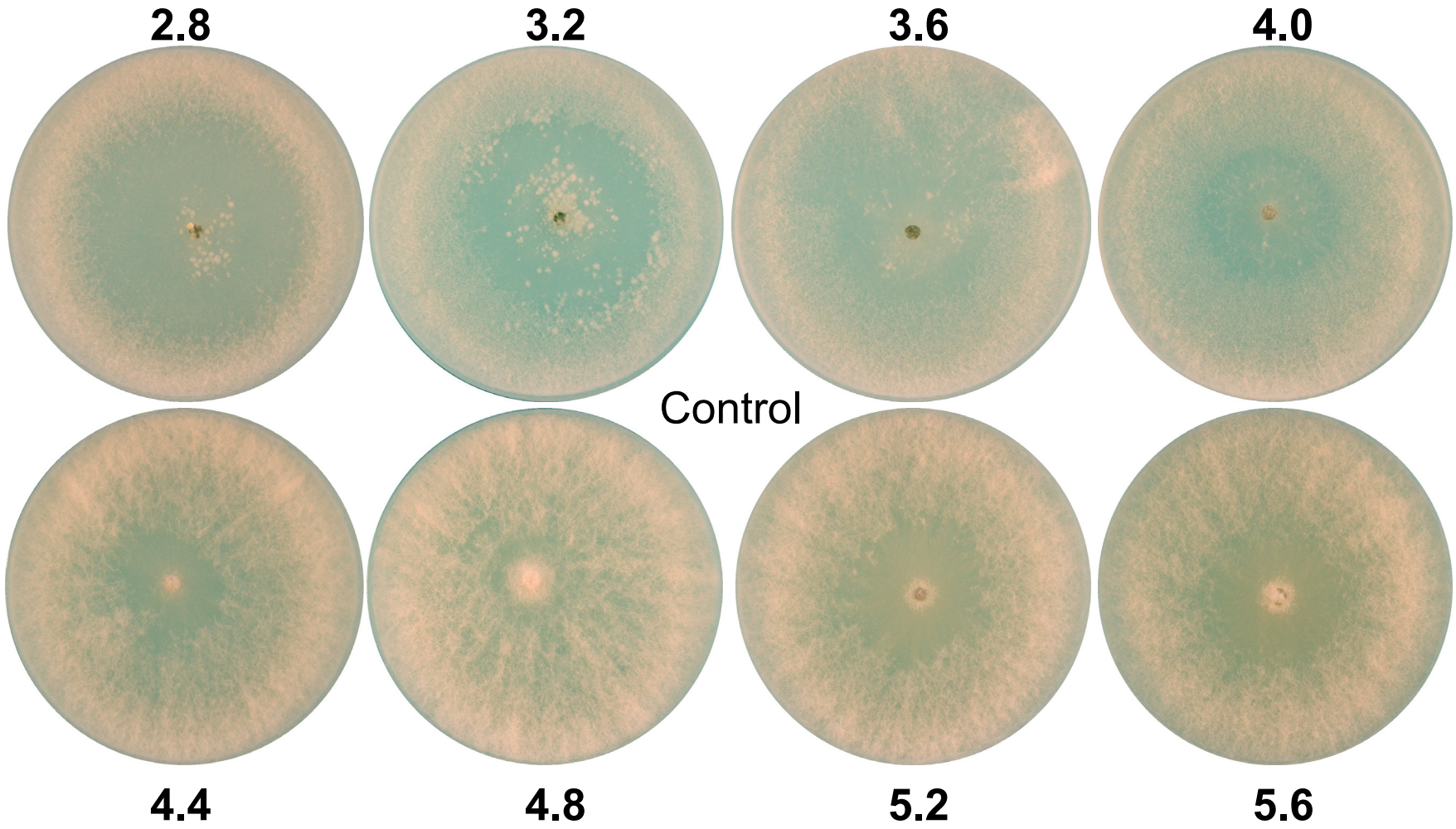


Figure 2.13A. *Trichoderma hamatum* injury induction experiment (control plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Cultures were grown for 6 d in total darkness.

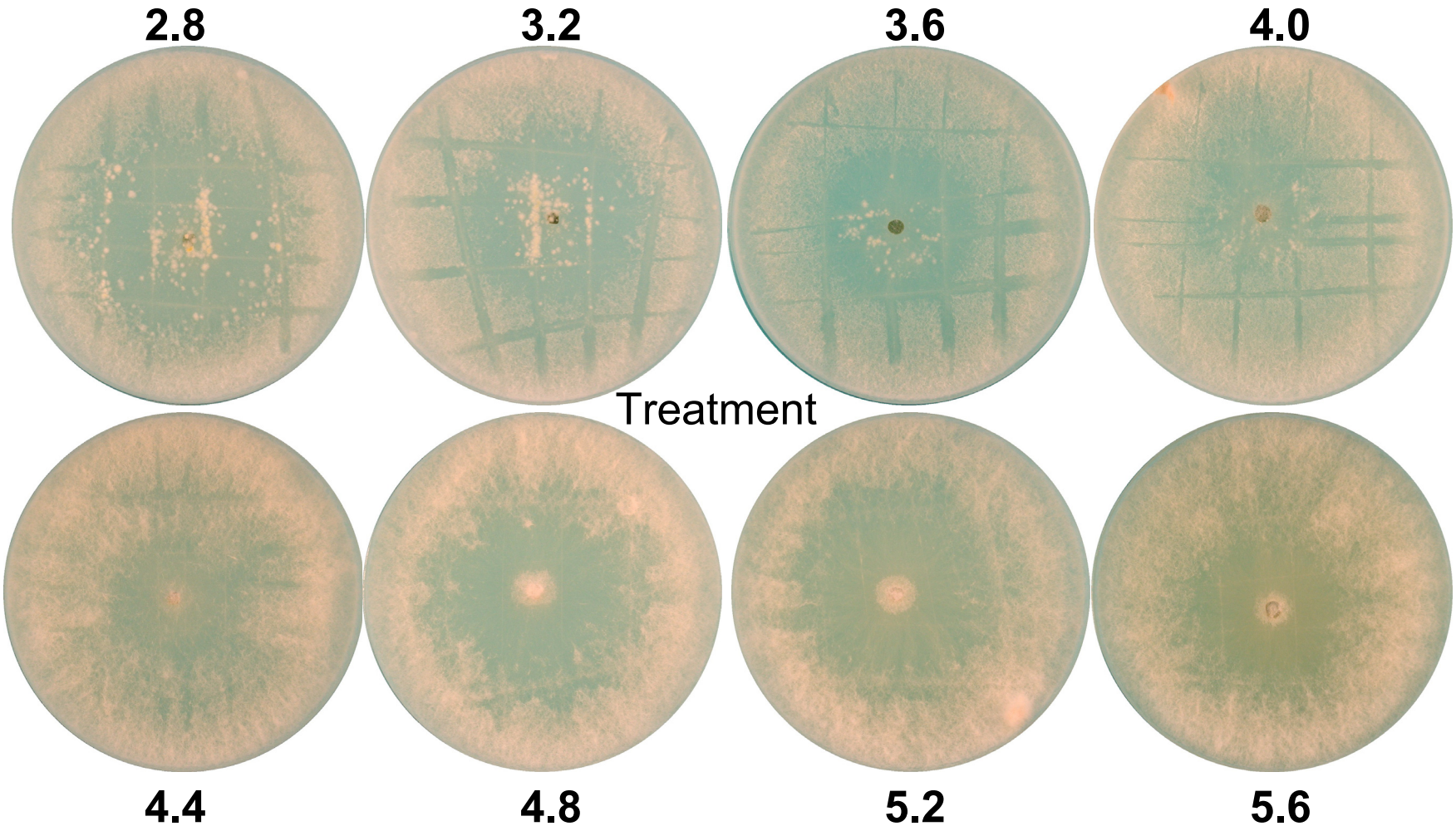


Figure 2.13B. *Trichoderma hamatum* injury induction experiment (treatment plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Cultures were grown for 6 d in total darkness. Plates were grown for 72 h in total darkness, then injury-induced and grown a further 72 h in total darkness.

2.7.6 Growth of *T. atroviride* at pH 5.8 to 7.8

The stress morphology observed on the *T. atroviride* plates in 2.7.5.1 was on agar buffered from 5.2 to 5.6 suggesting it may be a characteristic of growth at elevated pH levels. To test this hypothesis *T. atroviride* was grown for 5 days in total darkness on MM with glutamine phosphate/phosphate buffered from 5.8 to 7.8 (Figure 2.14). The stress morphology intensified and the growth rate slowed as the pH rose.

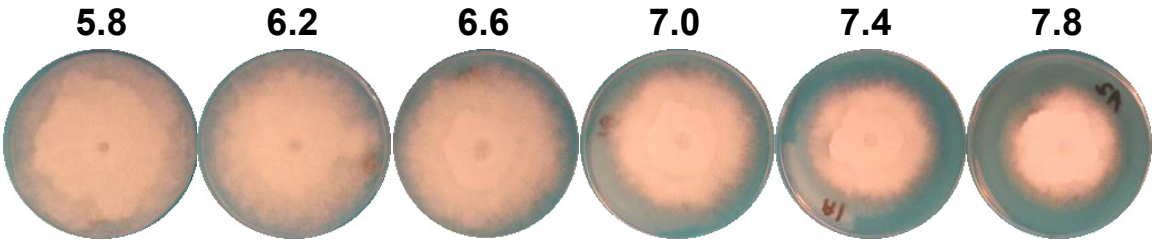


Figure 2.14. Growth of *T. atroviride* LU298 on pH buffered MM with glutamine.

2.8 Discussion

2.8.1 Conidiation Varies between Isolates on Nitrogen-amended Agar

The degree of variation in conidiation pattern differed greatly between isolates, as did the capacity to respond to conidiation stimuli. *Trichoderma hamatum* failed to conidiate in all nitrogen experiments, *T. virens* conidiated in response to stimuli in one out of eight experiments, *T. atroviride* in five, *T. harzianum* in seven and *T. asperellum* conidiated in response to both light and injury stimuli in every experimental combination tested. The intense and clear conidial responses observed in all experiments to date by *T. asperellum* (formerly *T. viride*) were not unexpected as isolates of this species have been used extensively as models for photoconidiation in *Trichoderma* (Chapter 1). In light of the variation in response to conidiation stimuli demonstrated for the other species of *Trichoderma*, it is not surprising that *T. viride* was chosen and routinely used for such studies. The differential response in this study highlights the need for research across the *Trichoderma* genus rather than just a few species and/or isolates.

2.8.2 Primary Nitrogen Promotes Conidiation in *T. asperellum* and *T. harzianum*

When high amounts of glutamine or urea were present in the medium as the sole nitrogen source, *T. asperellum* (formerly *T. viride*) produced a disk of conidia in response to a single exposure, whereas a photoconidiation ring was produced from cultures grown in the presence of KNO_3 as the nitrogen source. Amino and ammonium based primary nitrogen sources are routinely used to induce nitrogen catabolite repression in filamentous fungi and growth on KNO_3, or other nitrates strongly induces nitrogen derepression (Marzluf, 1997; ter Schure *et al*., 2000). Equimolar amounts of nitrogen were used in the glutamine, urea and KNO_3 experiments, therefore the amount of total N and C:N ratio were identical and both buffered and unbuffered media were used to account for variation due to pH changes. Any morphological differences between cultures grown on different nitrogen sources were assumed, therefore, to be due to the nitrogen status of the medium. The two photoconidiation phenotypes observed under repressing and derepressing conditions led to the hypothesis that: *T. asperellum* cultures under nitrogen catabolite repression (NCR) photoconidiate in a disk, whereas cultures in which repression is relieved conidiate in a ring in response to light. On potato-dextrose agar (PDA) *T. asperellum* photoconidiated in a ring, which, according to our hypothesis, suggested PDA invoked nitrogen derepression in this isolate. When incremental amounts of glutamine were added to the PDA to invoke nitrogen repressing conditions, the conidial pattern changed from a ring to a disk. The graduation from a ring to a disk supports the assertion that *T. asperellum* cultures grown on PDA were nitrogen derepressed, however as the glutamine concentration rose, colony growth decreased suggesting toxicity. The possible toxicity may have affected conidiation.

Potato-dextrose agar is a rich complex medium containing both primary and secondary nitrogen, though the exact nature and amounts of each source are not defined. It would appear, however, that the primary nitrogen levels in PDA were insufficient to invoke NCR in the entire *T. asperellum* colony. All previous studies on photoconidiation in *T. viride* have reported the production of a single ring of conidia in response to light (Gutter, 1957; Gressel & Galun, 1967; Chapter 1), which suggests that in those studies NCR was also relieved. The medium most often used to demonstrate photoconidiation is PDB or PDA, often amended with small amounts of extracts such as yeast extract and casein hydrolysate. These additives are rich in nitrogen, however the amount added

likely provided insufficient primary nitrogen to induce repression. In our experiments with PDA amended with glutamine, the production of a conidial disk occurred at high glutamine levels which reduced growth dramatically, whereas in the experiments described in Chapter 1 all cultures grew well.

In response to light, *T. harzianum* produced a disk of conidia when glutamine or urea was the sole nitrogen source and a ring when grown on KNO_3, however unlike *T. asperellum* a clear response was only observed on the unbuffered experiments. Buffering of the medium dramatically decreased the growth rate of the *T. harzianum* cultures such that at the time of light exposure colonies on buffered medium were one third the size of the unbuffered medium. The reduced growth rate made interpretation of the buffered results difficult, however in the unbuffered experiments *T. harzianum* photoconidiated in a disk under nitrogen repressing conditions and a ring under derepressing conditions, which suggested photoconidiation was regulated in a similar fashion to *T. asperellum*.

When primary nitrogen was scarce, or absent and KNO_3 was the sole nitrogen source, conidiation in response to light was constrained to the perimeter cells only, whereas in the presence of abundant primary nitrogen photoconidiation occurred in a disk. Rather than two phenotypes, the disk and the ring may represent degrees of the same response. This was evidenced by the transition from a ring to a disk when NCR was induced in *T. asperellum*. In *T. atroviride*, photoinduction of conidiation requires the Blue-Light Regulators BLR-1 and BLR-2 (Casas-Flores *et al.*, 2004, 2006; Section 1.5.1). Conservation of *blr-1*/*blr-2* with other filamentous fungi, such as *wc-1*/*wc-2* in *N. crassa* suggests BLR-1/BLR-2 may regulate blue-light induced conidiation in all *Trichoderma* spp.. Based on the assumption that *blr-1*/*blr-2* orthologues also operate in *T. asperellum*, then it is possible that under nitrogen derepressing conditions conidial development mediated by the BLR-pathway is blocked in the centre of the colony and induced at the perimeter. Further, as primary nitrogen becomes more abundant in the medium, BLR-mediated photoconidiation activity extends from the perimeter of the colony inwards until all cells are active and presumably absolute NCR is established. Based on these studies, it is hypothesised that in *T. asperellum* and *T. harzianum* the nitrogen catabolite repression status of the environment cross-regulates competency to conidiate in response to light as mediated by BLR-1/BLR-2.

The transition from a photoconidiation ring to a disk in response to increasing levels of glutamine suggested that depending on the nitrogen status of the medium, NCR was invoked differentially across the fungal colony. It also suggested that NCR was invoked at the perimeter of derepressed cultures. A mechanism through which NCR could be established in the perimeter of derepressed cultures is the active translocation of amino acids through cytoplasmic connections between hyphal cells. In the absence of sufficient nitrogen, fungal cells can assimilate nitrates by reduction to ammonia which is then converted to glutamate or glutamine, thus primary nitrogen metabolism can occur when nitrates are the sole nitrogen source (Wiame *et al.*, 1985; Marzluf, 1997). Hyphal cells store and translocate amino acids in a system which allows for the regulation of developmental genes by controlling the intracellular N-status (Watkinson, 1999). Movement of nitrogen compounds, as well as other nutrients occurs around the fungal colony and in some cases is bidirectional (Olsson, 1999; Watkinson, 1999). In addition, nutrients have been shown to move in a defined path through the colony suggesting differentiation, however no visible changes in mycelial structure were present along these pathways. Under conditions of nitrogen repression in *T. asperellum* and *T. harzianum*, both primary nitrogen scavenged from the environment and intracellular reserves generated through the reduction of nitrates could be translocated to the colony perimeter, thus maintaining NCR in the hyphal front, which in turn promoted photoconidiation.

In *T. atroviride*, sudden nitrogen starvation has been demonstrated to induce a diffuse disk of conidia from colonies grown on filter paper, which suggested that nitrogen derepression promoted starvation-induced conidiation (Casas-Flores *et al.*, 2006). Nitrogen starvation stimulated expression of *N. crassa* conidiation genes, which clearly showed a link between nitrogen derepression and regulation of conidiation (Sokolovsky *et al.*, 1992). Our studies and these results are contradictory, since it would appear that depending on the light conditions both NCR and derepression can stimulate conidiation. Genes under the influence of nitrogen catabolite repression are repressed in the presence of and induced in the absence of, primary nitrogen sources and this control is highly regulated often involving additional pathway-specific regulators (Marzluf, 1999). If induction of conidiation is under the control of NCR, it would seem highly unlikely that conditions inducing NCR also stimulated conidiation in these experiments. Nevertheless photoconidiation was induced in cells which were subject to nitrogen catabolite repression at the time of light exposure.

Understanding how NCR stimulated competency of hyphal cells to photoconidiate in *T. asperellum* will require further studies, however on the basis of these experiments and observations in the literature two mechanisms can be postulated:

I. NCR may have stimulated photoconidiation through Protein Kinase A.

In *Saccharomyces cerevisiae*, the addition of amino acids or ammonium to nitrogen-starved cells grown in the presence of glucose as the carbon source activates the PKA pathway and this is independent of cAMP (Thevelein *et al.*, 2005; Van Nuland *et al.*, 2006). Activation of the PKA pathway by glutamine is mediated by the amino acid permease, Gap1, whereas ammonium stimulation of PKA activity involves the Mep ammonium permeases (Donaton *et al.*, 2003; Van Nuland *et al.*, 2006). These permeases act as both sensors and transporters of amino acids and ammonium. Putative orthologues of Gap1 have been identified in the genomes of filamentous fungi (http://www.ncbi.nlm.gov/Genbank) and blast analysis of the Gap1 protein sequences from *S. cerevisiae*, *Aspergillus fumigatus* and *Gibberella zeae* against the *T. reesei* genome revealed a putative orthologue with high similarity to both yeast and filamentous fungi sequences (this study, data not shown). High PKA activity has also been demonstrated to be involved in regulation of BLR-inducible genes in *T. atroviride* (Casas-Flores *et al.*, 2006; Section 1.5.4.3). Mutational analysis of the PKA regulatory subunit suggested that high PKA activity was essential for expression of rapid-light BLR-inducible genes, whereas low PKA activity was essential for conidial development. If glutamine and urea promoted PKA activity in *T. asperellum*, then by inference, growth under NCR-inducing conditions also promoted the induction of rapid-light BLR-inducible genes. Further, amino acid permeases and other nitrogen utilisation genes are also light inducible (Alfredo Herrera-Estrella, pers. comm.). The stimulation of PKA activity by primary nitrogen sources provides a mechanism by which photoconidiation could be promoted by NCR in *T. asperellum*.

II. Derepression may be mediated through the actions of nitrate reductase.

If NCR stimulated PKA activity and expression of rapid blue-light genes, and nitrogen derepression and low PKA activity are required for conidial development, then a mechanism must exist whereby derepression is induced in cells which have perceived the light. In *N. crassa* the nitrate reductase gene, *nit-3*, is expressed in a circadian fashion under conditions inducing derepression (Christensen *et al.*, 2004). Nit-3 reduces nitrates to nitrites, which are further reduced to ammonium ions and then

converted to glutamine and glutamate. When the level of glutamine inside the cell reaches a critical level *nit-3* expression is turned off, thereby forming a negative feedback loop. This rhythm is endogenous and self-sustaining and is also independent of the WCC/FRQ complex, though the amplitude of the rhythm is affected in Δ*frq* and Δ*wc-2* mutants (Christensen *et al*., 2004). During growth on limited primary nitrogen, *nit-3* oscillation would result in the establishment and relief from NCR as the colony expands. Alternating nitrogen repressing and derepressing conditions provide a plausible mechanism which could account for the production of a ring in *T. asperellum* during growth on KNO_3. A flavin molecule is contained within the Nit-3 protein, which has led to speculation that Nit-3 itself may act as a blue-light receptor, however there is no evidence as yet to support this (Ninnemann, 2001). Induction of *nit-3* by light and the resulting intracellular nitrogen derepression provides a mechanism by which light induction could stimulate derepression thus allowing conidial development to occur.

2.8.3 The Ambient pH Strongly Influences Conidiation in *T. atroviride* and *T. hamatum*

Rings of conidia were produced in *T. atroviride* in response to light regardless of nitrogen source, which suggested that in the nitrogen experiments photoconidiation was differently regulated in this isolate compared with the *T. asperellum* and *T. harzianum* isolates under study. The ring was thicker and denser in the primary nitrogen experiments compared with KNO_3, which suggested some promotion of photoconidiation by NCR-inducing conditions, however the increase in conidiation may be due to the considerably denser growth on primary nitrogen-amended agar. The absence of a disk in *T. atroviride* suggested that either NCR-inducing conditions did not cross-regulate competency to photoconidiate and/or, another regulatory factor constrained competency to photoconidiate in this isolate. The photoconidiation ring produced on PDA had a partially filled in appearance (Section A, Figure 2.2C), which suggested that like *T. asperellum*, conidiation in response to light is not always constrained to the perimeter. Conidiation in response to light may have been constrained by the ambient pH of the medium. On the buffered medium amended with glutamine or urea the *T. atroviride* colony failed to photoconidiate and appeared stressed. As the pH was increased on MM with glutamine from 5.6 to 7.8 this stress morphology became more pronounced. On the unbuffered medium the stress morphology was absent. In addition, at the time of light induction the agar under the colony front of the unbuffered MM with glutamine plates was pH 4.2 whereas the uncolonised agar was at pH 5.8,

which suggested acidification of the agar by activities of the hyphal front. These observations suggested that either conidiation was inhibited by the buffering of the medium, or pH 5.4 was too high for photoconidiation. In contrast to *T. atroviride* LU298, buffering had no detrimental effect on *T. atroviride* IMI206040, which grew well on glutamine-amended medium buffered to pH 5.6 (Alfredo Herrera-Estrella, pers. comm.). Both isolates have been identified by sequencing of the ITS region, which suggests that the different growth responses on the buffered glutamine media were isolate-specific.

Conidiation in response to light was absent in both *T. hamatum* and *T. virens* in all nitrogen experiments suggesting that either both nitrogen sources are required or PDA contains something necessary for photoconidiation in these two isolates, which is lacking in minimal medium. The lack of conidiation in *T. hamatum* was not surprising as this isolate responded poorly to a single light-dose on PDA but was unexpected for *T. virens* as this isolate produced a clear conidiation ring on PDA (Section A). The explanation for the lack of conidiation in both these isolates is unknown, however, like *T. atroviride*, other factors such as the ambient pH of the medium may have constrained the response.

In response to mycelial injury *T. atroviride* conidiated on unbuffered glutamine and urea and both buffered and unbuffered KNO_3, which suggests that as with photoconidiation, primary nitrogen medium buffered to pH 5.4 was not optimal for injury-induced conidiation. In addition, on the unbuffered primary nitrogen and KNO_3 plates, conidiation in response to injury occurred in a ring at what was the perimeter of the colony when injured. On PDA, conidiation occurred across the colony in response to injury, though conidial pigmentation was more mature at the perimeter. Together these results suggested that in the presence of a single nitrogen source, conidiation in response to injury was constrained to the perimeter. On the glutamine plates the agar under the perimeter at injury was at pH 4.8, while in the centre of the colony it was at pH 6.5, which, as in the photoconidiation experiment, suggested acidification of the agar by activities of the hyphal front. Prolific conidiation at the site of acidification suggested low pH strongly favoured injury-induced conidiation. On PDA buffered from pH 2.8 to 5.4, *T. atroviride* conidiated in response to injury in a pH dependent manner, which clearly demonstrated that the ambient pH had a major regulatory influence on injury-induced conidiation. In contrast, no conidiation was observed at any pH on the

glutamine medium, which strongly suggested that injury-induced conidiation in *T. atroviride* LU298 was blocked on buffered medium when glutamine is the sole nitrogen source.

On buffered PDA, *T. hamatum* conidiated in response to injury in a pH-dependent manner similar to *T. atroviride*. No response to injury was observed in all previous experiments, suggesting that this isolate may not be capable of an injury response. However, injury-induced conidiation was clearly observed at pH 2.8 and 3.2, which suggested that ambient pH may have constrained conidiation in previous PDA experiments.

It is clear from these experiments that the ambient pH cross-regulated conidiation in response to injury in *T. atroviride* and *T. hamatum* colonies. It is not known if the ambient pH cross-regulates photoconidiation as well in these isolates, however both blue-light and injury induced conidiation were constrained to the more acidic region of the *T. atroviride* colony on unbuffered glutamine medium, which suggested competency to conidiate in response to both stimuli was constrained by the ambient pH. Indeed, a dependence on low pH for conidiation in response to injury was clearly demonstrated using buffered PDA. The underlying pathways involved in injury-induced conidiation are unknown, however like light and starvation-induced conidiation, cAMP signalling is likely to be involved. In *T. viride*, light-induced intracellular acidification has been postulated to contribute to cAMP production, which in turn stimulated conidiation (Grešik *et al.*, 1991). A link between intracellular acidification and increases in cAMP has been demonstrated in *Aspergillus niger* and *Saccharomyces cerevisiae* (Gradišnik-Grapulin & Legiša, 1997; Caspani *et al.*, 1985). Further, in *S. cerevisiae* a reduction in the pH of the medium from 8.5 to 3.5 caused a concomitant drop in intracellular pH and an increase in the level of cAMP (Caspani *et al.*, 1985). If the low pH of the agar under the hyphal front is associated with intracellular pH, then it is likely production of cAMP was also stimulated in these cells, which may have promoted conidiation.

In summary, we have demonstrated that NCR promotes competency to photoconidiate in hyphal cells of *T. asperellum* and *T. harzianum* resulting in the formation of a disk, whereas under derepressing conditions competency decreased to a ring. In contrast, no regulatory link between NCR and photoconidiation was suggested in *T. atroviride*, however injury-induced conidiation was clearly pH-dependent. The link between low

pH and conidiation may relate to cAMP signalling, however the mechanism by which NCR stimulated competency to photoconidiate in *T. asperellum* and *T. harzianum* is unknown.

SECTION C
THE INFLUENCE OF AMBIENT pH ON PHOTOCONIDIATION

2.9 Introduction

In the previous two sections, it was demonstrated that conidiation in response to a single light burst in *Trichoderma* spp. is not restricted to the colony perimeter, as had been previously thought. It was also demonstrated that competency to photoconidiate is dependent on the metabolic state of the cell, rather than metabolic rate and age, and this is dependent on the environment. Photoconidiation was cross-regulated by the nitrogen status of the cell in *T. asperellum* and *T. harzianum* and injury-induced conidiation was clearly pH-dependent in *T. atroviride* and *T. hamatum* (Section B). The relationship between ambient pH and photoconidiation was not investigated, however, intracellular acidification has been shown to be associated with photoconidiation in *T. viride* (Gresik *et al.*, 1991). These authors proposed that intracellular acidification, which occurred as a result of light exposure, contributed to the observed rise in cAMP, which in turn promoted conidiation (Gresik *et al.*, 1991; Section 1.5.4.1). In this study, the influence of ambient pH on photoconidiation was investigated in *T. atroviride* and *T. hamatum*. On primary nitrogen medium the *T. harzianum* colony grew in a spoke-like pattern, though only when the medium was buffered, which clearly demonstrated a relationship between buffering of the medium and colony morphology (Section B). To further explore this relationship, the influence of ambient pH on growth and photoconidiation was also investigated in *T. harzianum*.

Given the role ambient pH plays in many different regulatory circuits, it can be difficult to design experiments which focus solely on pH. Whilst a clear relationship between injury-induced conidiation and pH was observed in the PDA experiments, no conidiation was observed in *T. atroviride* and *T. hamatum* on buffered medium when glutamine or urea were the sole nitrogen sources, which suggested cross-talk with nitrogen catabolite repression (Section B). In order to investigate the influence of ambient pH only, it has been considered acceptable to manipulate the system to minimise the influence of other regulatory pathways (Penalva & Arst, 2004). For this reason, PDA, which doesn't induce nitrogen catabolite repression, was used as the agar medium on which the influence of ambient pH on photoconidiation was investigated. Light-induction of conidiation rather than injury-induction was used as this is a well studied phenomenon in some species of *Trichoderma*, thus providing substantial

comparative data, whereas injury-induced conidiation has only recently been described. Based on the literature and observations from the previous sections, we hypothesise that light induction is pH-dependent in *T atroviride* and *T. hamatum* and that the ambient pH influences colony morphology in *T. harzianum.*

2.10 Materials and Methods

2.10.1 Photoconidiation in *T. atroviride* and *T. hamatum* at pH 2.8 to 5.2 on pH-buffered PDA

PDA was prepared as described in 2.6.4. Conidial suspensions prepared as described in 2.2.2, were inoculated to PDA and the plates were incubated and photoinduced as described in 2.2.3. The pH values of the plates were measured prior to inoculation and at the time of light exposure as described in 2.6.3. Control plates were incubated in total darkness for the duration of the experiment. Two plates were inoculated per treatment and the experiment was repeated twice.

2.10.2 Photoconidiation in *T. atroviride* and *T. hamatum* at pH 2.8 to 5.2 on unbuffered PDA

PDA was prepared essentially as described in 2.6.4 except that PDB was dissolved in water rather than citrate/phosphate buffer. Conidial suspensions, prepared as described in 2.2.2, were inoculated to PDA and the plates were incubated and photoinduced as described in 2.2.3. The pH values of the plates were measured prior to inoculation and at the time of light exposure as described in 2.6.3. Control plates were incubated in total darkness for the duration of the experiment. Two plates were inoculated per treatment and the experiment was repeated twice.

2.10.3 Effect of Culture Age on Photoconidiation at pH 2.8 in *T. atroviride*

PDA buffered to pH 2.8 was prepared as described in 2.6.4 except that sterilised agar was poured into 150 mm Petri dishes instead of the standard 90 mm. In the first experiment, conidial suspensions, prepared as described in 2.2.2, were inoculated to PDA and the plates were incubated unsealed in total darkness at 25°C. Plates were exposed to blue-light at 24, 48, 72, 96, 120 or 144 h and then incubated a further 72 h in total darkness.

All experiments in the chapter to date have used conidial suspensions as the inoculum source to ensure that all cultures are naïve to light. In this way the controls were true dark-grown cultures and the treatment plates received a controlled light exposure only. To test whether the light response observed in 2.11.3.1 is independent of the starting material, the experiment was repeated using mycelial plugs as inoculum. In the second experiment, mycelial plugs from 2 d old *T. harzianum* cultures grown on PDA were used as inoculum. Separate control plates were incubated for an equivalent length of time in total darkness. Two plates were inoculated per treatment and both experiments were repeated once.

2.10.4 Effect of Culture Age on Injury-induced Conidiation at pH 2.8 in *T. atroviride*

PDA buffered to pH 2.8 was prepared as described in 2.6.4 and 2.10.3. Conidial suspensions, prepared as described in 2.2.2, were inoculated to PDA and the plates were incubated unsealed in total darkness at 25°C. Colonies were injured as described in 2.2.4 at 24, 48, 72, 96, 120 or 144 h and then incubated a further 72 h in total darkness. Separate control plates were incubated for an equivalent length of time in total darkness. Two control plates and three injury plates were inoculated per treatment. The experiment was not repeated.

2.10.5 Light Response of *T. harzianum* at pH 2.8 to 5.2

Media was prepared as described in 2.10.1 and 2.10.2. In the first experiment, buffered PDA was inoculated with a conidial suspension and photoinduced as described in 2.2.2 and 2.2.3. In the second experiment, buffered and unbuffered PDA was inoculated with mycelial plugs from 2 d old *T. harzianum* cultures grown on PDA, and photoinduced at 48 h as described in 2.2.3. In addition, the buffered PDA was inoculated with a conidial suspension and incubated under the same conditions for 6 d. The pH values of the plates were measured as described in 2.2.4.3. Control plates were incubated in total darkness for the duration of the experiment. Two plates per treatment were inoculated and both experiments were repeated once.

2.11 Results

2.11.1 Photoconidiation in *T. atroviride* and *T. hamatum* at pH 2.8 to 5.2 on pH-buffered PDA

2.11.1.1 *Trichoderma atroviride*

The influence of ambient pH on photoconidiation in *T. atroviride* was investigated using PDA buffered from pH 2.8 to 5.2. Conidiation occurred in a pH-dependent manner and the strongest response was at the lowest pH, as observed in the injury induction experiments (2.7.5.1). Immature conidiation was observed in the pH 2.8 control plates in a 25 mm diameter zone at the centre of the colony (Figure 2.15A). The size of the zone and the intensity of conidiation rapidly diminished as the pH rose and only a few hyphal tufts were observed at pH 4.0. At pH 5.2, the colony morphology began to change and resemble the previously described stress morphology. In response to a single light exposure, *T. atroviride* produced a disk of profuse conidiation at pH 2.8 and this correlated with the colony margin at the time of light exposure (Figure 2.15B). The disks were predominantly green and immature at the margins. As the pH rose, the conidial maturity and intensity decreased and at pH 4.0 only a small cluster of immature conidia were observed at the centre of the plate. The pH 4.4 to 5.2 treatment plates were indistinguishable from their relative controls. The pH values of the plates at the time of light exposure did not deviate significantly from the starting point of the experiment.

2.11.1.2 *Trichoderma hamatum*

The influence of ambient pH on photoconidiation in *T. hamatum* was evaluated on buffered PDA within the same experiment as *T. atroviride* above and similarly conidiation occurred in a low pH-dependent manner. Sparse conidiation was observed at the centre of the pH 2.8 and 3.2 control plates and this was slightly more intense at pH 3.2 (Figure 2.16A). No conidia were discernible above pH 3.2 and, at pH 4.4 and above, the hyphal morphology began to alter. Conidiation in response to light was clearly observed on pH 2.8 and 3.2 only and as with the control plates this was more intense at pH 3.2 (Figure 2.16B). At pH 2.8, the conidia were colourless and at pH 3.2 they were colourless to yellow. Conidia were clustered around the centre of the plate and the margin of the conidial zone was considerably smaller in diameter than that of the colony at the time of light exposure. The pH 3.6 to 5.2 treatment plates were indistinguishable from their respective controls. The pH values of the plates at the time of light exposure did not deviate significantly from the starting point of the experiment.

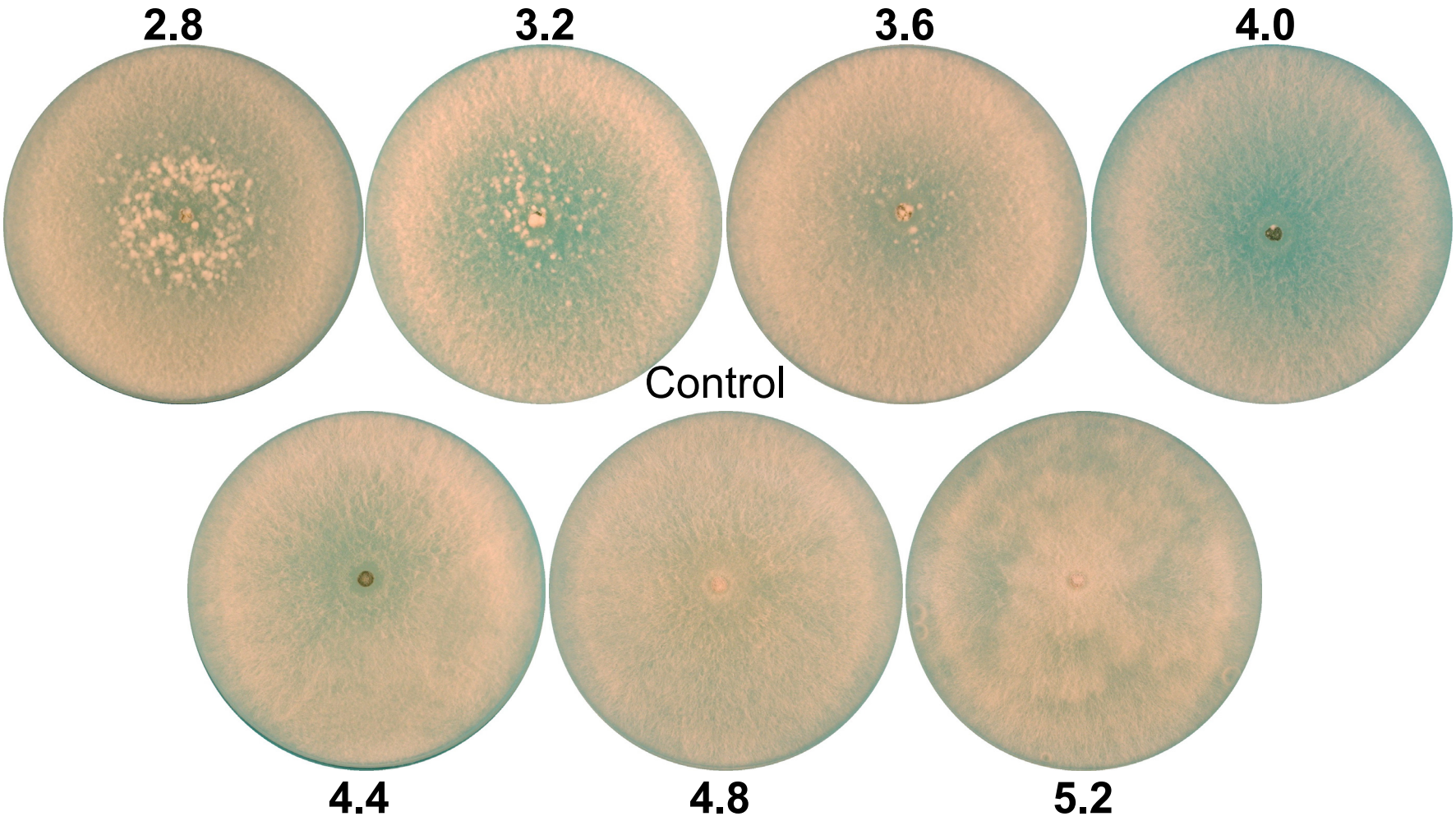


Figure 2.15A. *Trichoderma atroviride* photoconidiation experiment (control plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Cultures were grown for 5 d in total darkness.

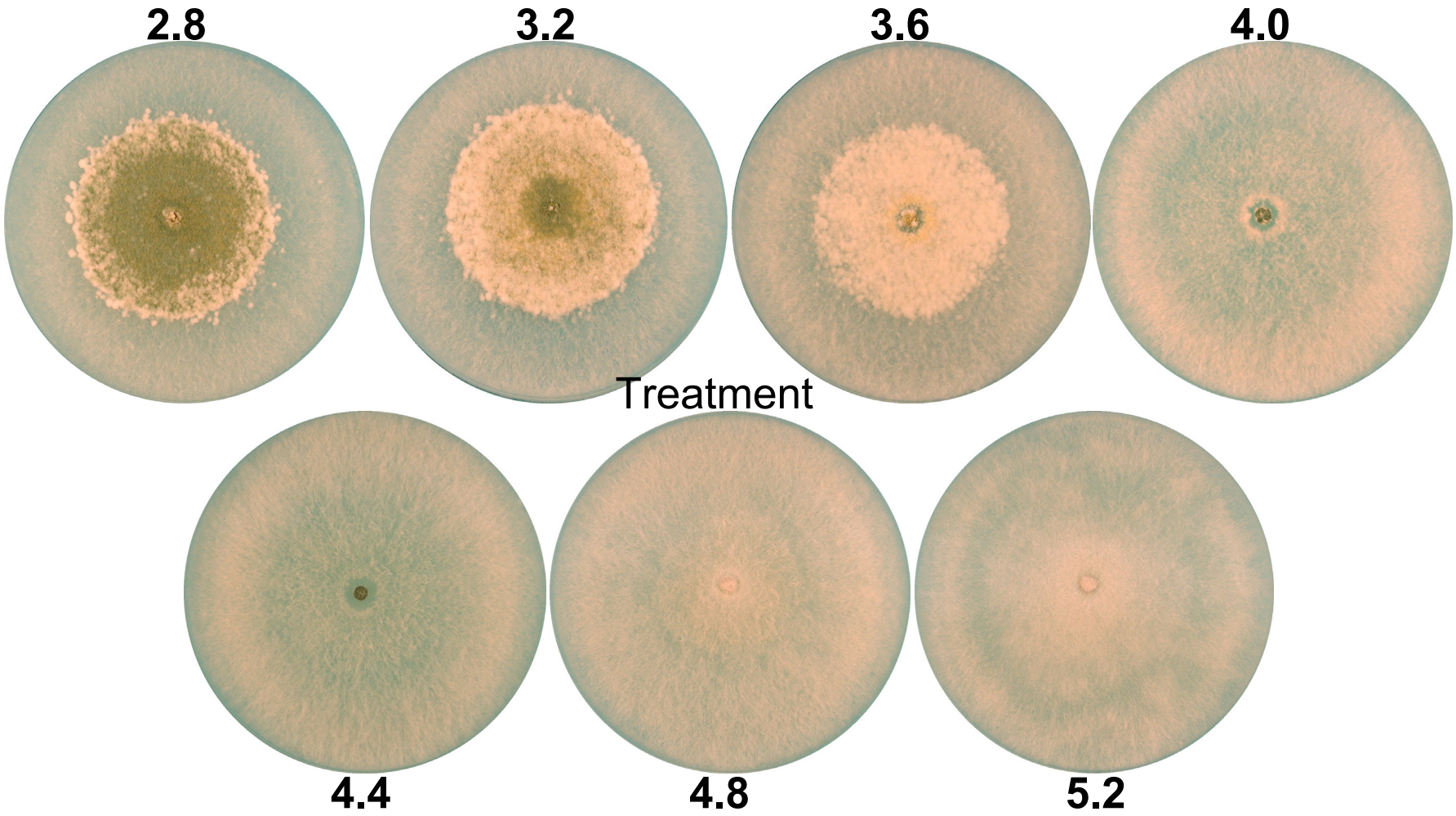


Figure 2.15B. *Trichoderma atroviride* photoconidiation experiment (treatment plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Cultures were grown for 48 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

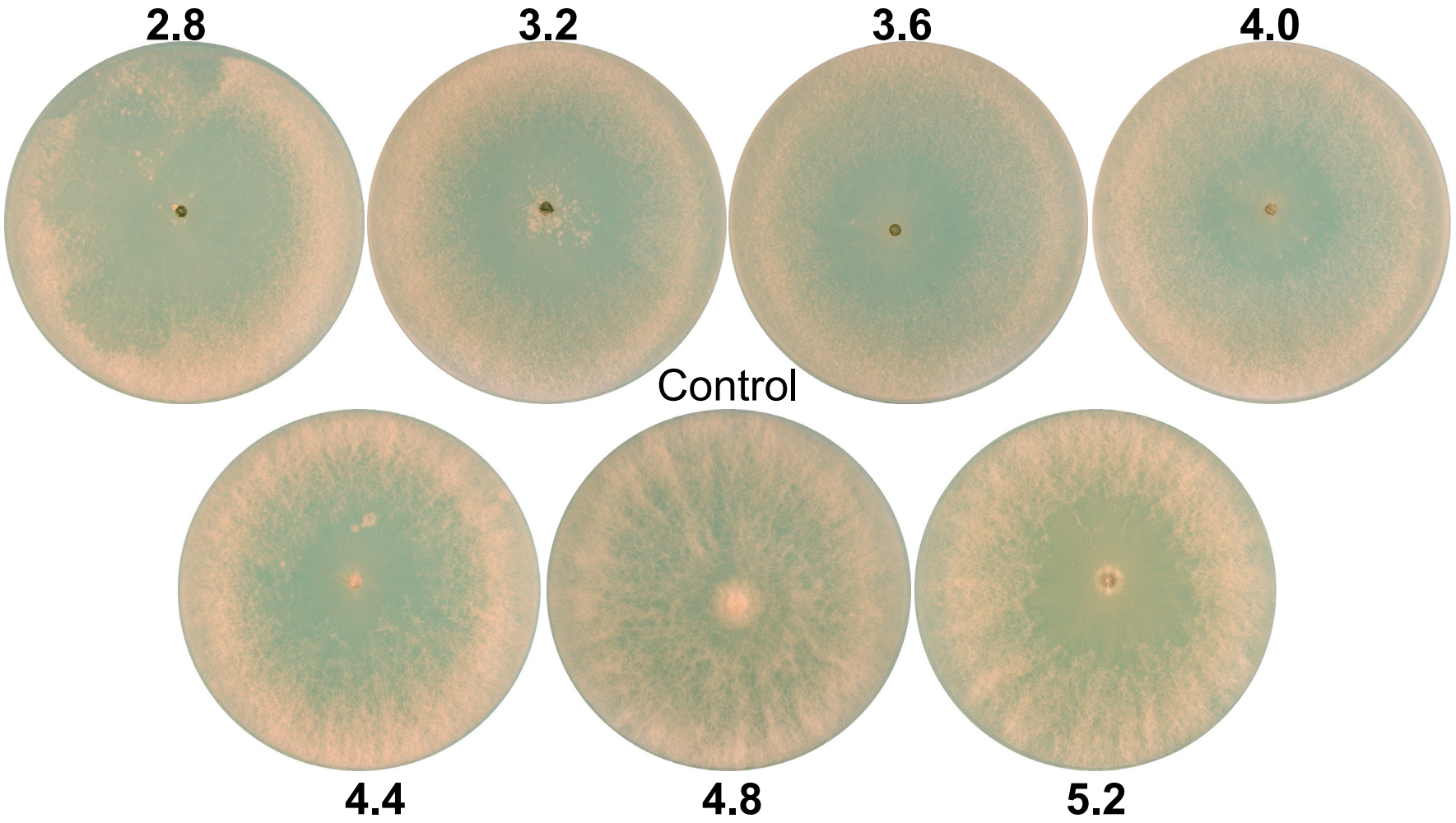


Figure 2.16A. *Trichoderma hamatum* photoconidiation experiment (control plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Cultures were grown for 5 d in total darkness.

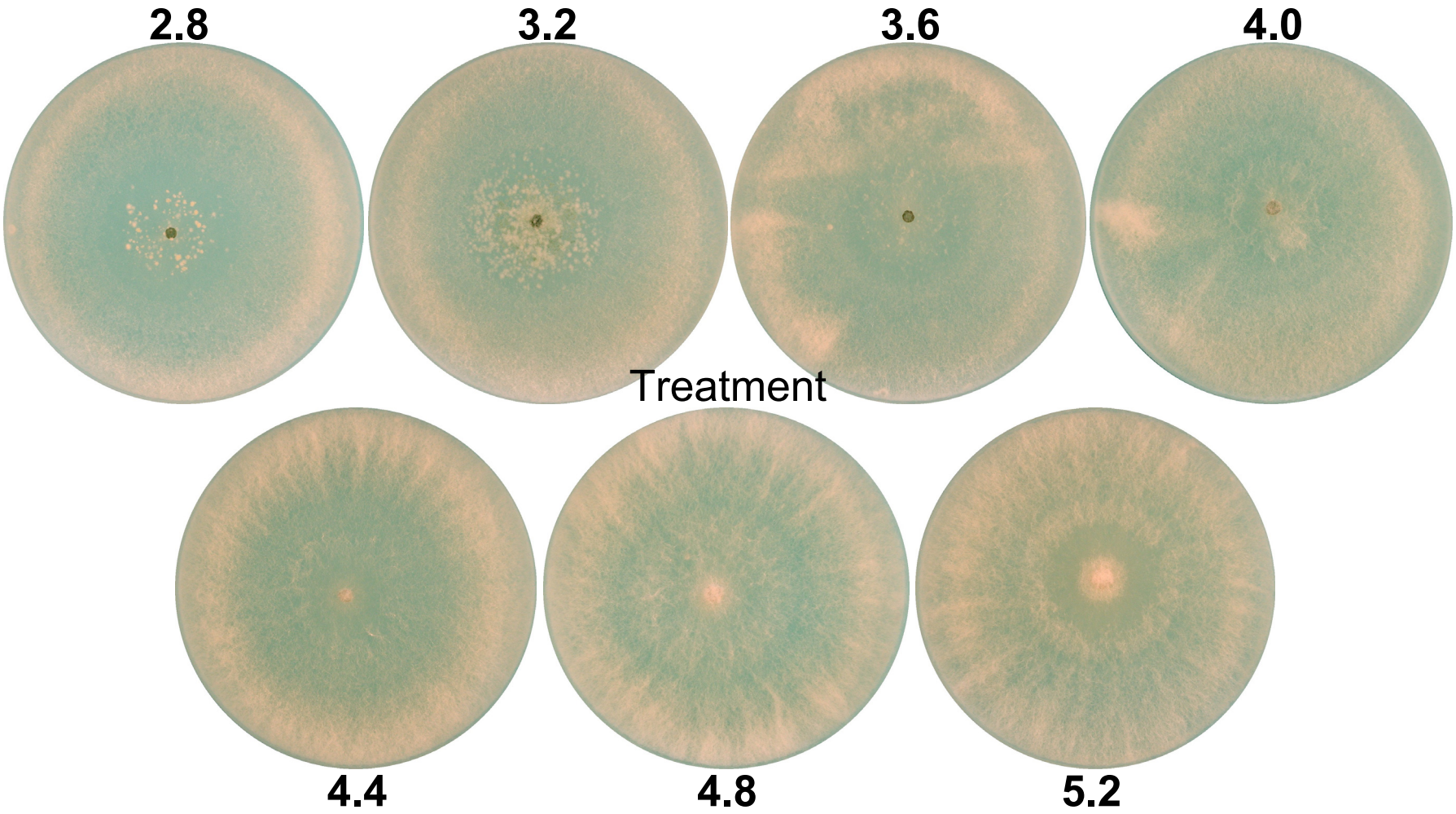


Figure 2.16B. *Trichoderma hamatum* photoconidiation experiment (treatment plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Cultures were grown for 48 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

2.11.2 Photoconidiation in *T. atroviride* and *T. hamatum* at pH 2.8 to 5.2 on unbuffered PDA

2.11.2.1 *Trichoderma atroviride*

The influence of ambient pH on photoconidiation in *T. atroviride* was also investigated using unbuffered PDA which had been acidified from pH 2.8 to 5.2. Similar to the buffered experiment, conidiation was observed on the control plates from pH 2.8 to 4.0, though it was more apparent between pH 3.2 and 3.6 (Figure 2.17A). Conidiation was immature, sparse and clustered around the centre of the plates. Hyphal growth was less dense than on the buffered medium and no changes in hyphal morphology were apparent as the pH rose. In contrast to the buffered experiment, light-induced conidiation was observed on all the treatment plates and this was most intense at pH 3.6 (Figure 2.17B). Conidia were produced in a disk and the edge of the disk correlated with the colony margin at the time of light exposure. Green pigmented conidia were observed from pH 3.2 to 4.4 and the most mature conidiation was observed at pH 3.6. From pH 4.4 to 5.2, conidiation was considerably less dense around the central inoculation site.

2.11.2.2 *Trichoderma hamatum*

The influence of ambient pH on photoconidiation in *T. hamatum* was evaluated in the same unbuffered PDA experiment as for *T. atroviride* above. Minimal conidiation was observed on all control plates clustered around the centre and was most prevalent from pH 3.2 to 4.4 (Figure 2.18A). This differed from *T. atroviride* in the same experiment where conidiation occurred only from pH 2.8 to 4.0 and from the *T. hamatum* buffered experiments in which conidiation only occurred at pH 2.8 and 3.2. Like *T. atroviride*, hyphal growth was less dense than on the buffered control plates. In response to light, conidiation occurred on all treatment plates and this was most intense at pH 2.8 and 3.2 (Figure 2.18B). Conidia were colourless to yellow in pigment and clustered around the centre and the diameter of the conidial zone was considerably smaller than that of the colony at the time of light exposure.

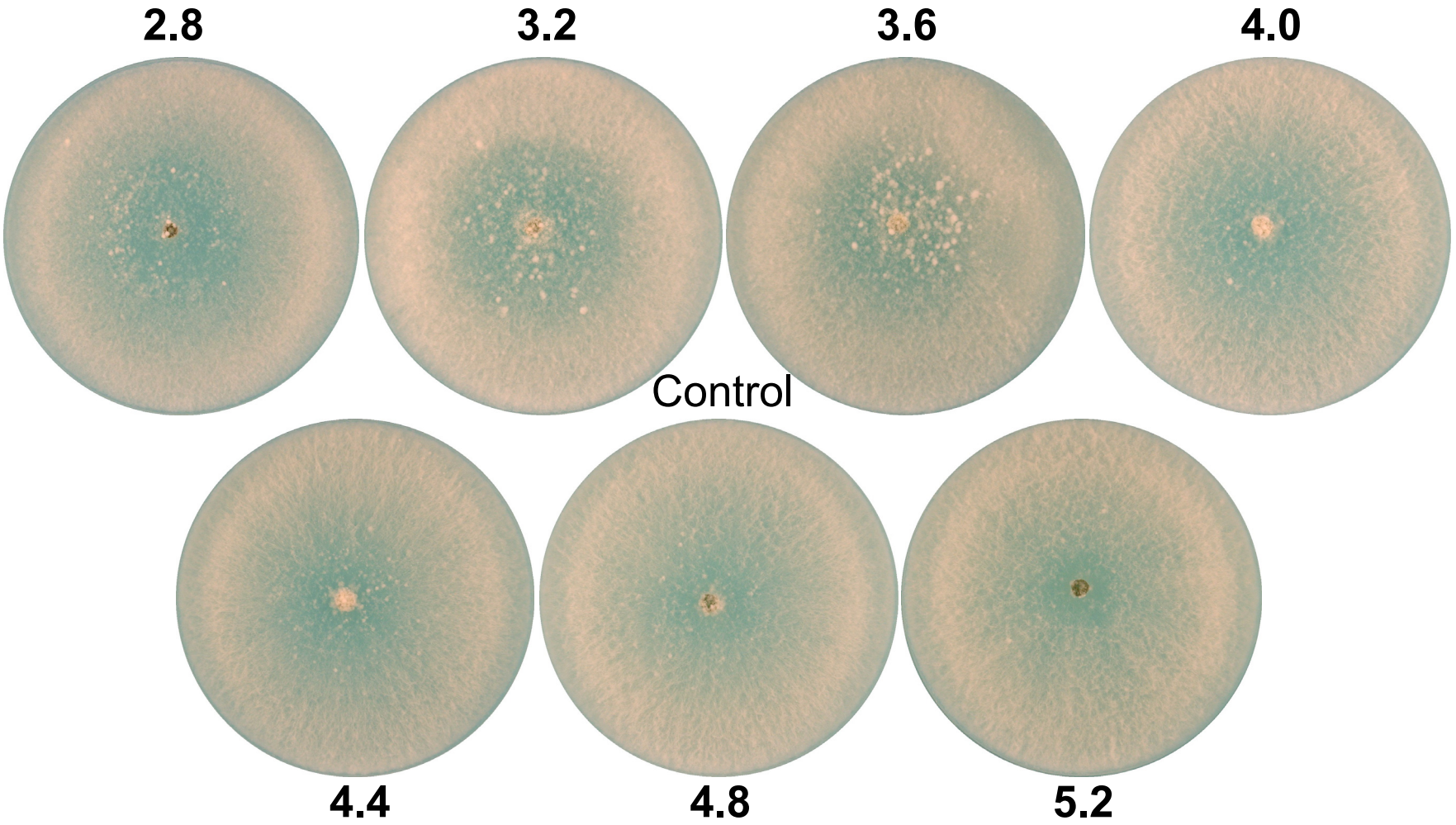


Figure 2.17A. *Trichoderma atroviride* photoconidiation experiment (control plates) on unbuffered, pH adjusted (2.8 to 5.2) PDA. Cultures were grown for 5 d in total darkness.

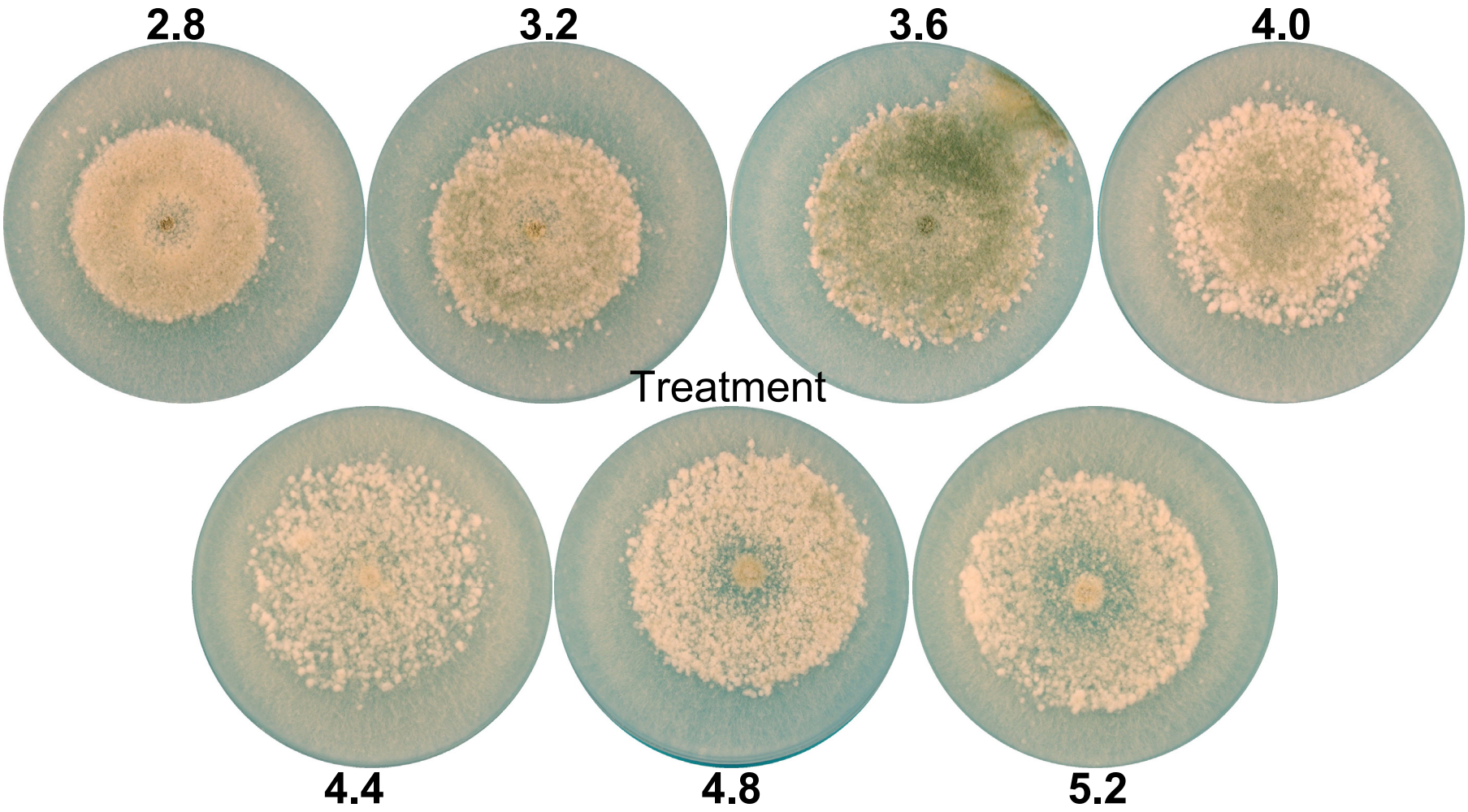


Figure 2.17B. *Trichoderma atroviride* photoconidiation experiment (treatment plates) on unbuffered, pH adjusted (2.8 to 5.2) PDA. Cultures were grown for 48 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

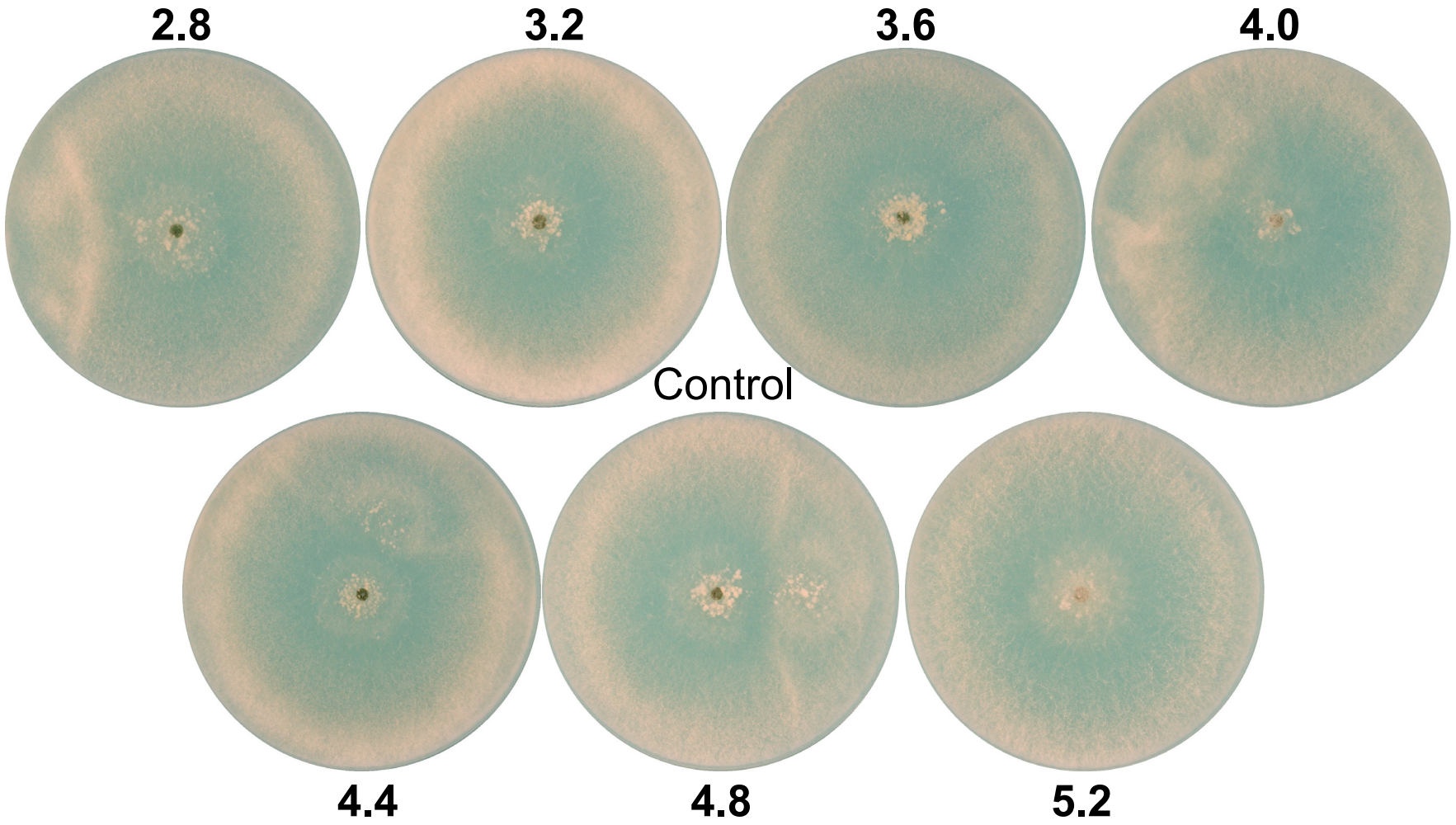


Figure 2.18A. *Trichoderma hamatum* photoconidiation experiment (control plates) on unbuffered, pH adjusted (2.8 to 5.2) PDA. Cultures were grown for 5 d in total darkness.

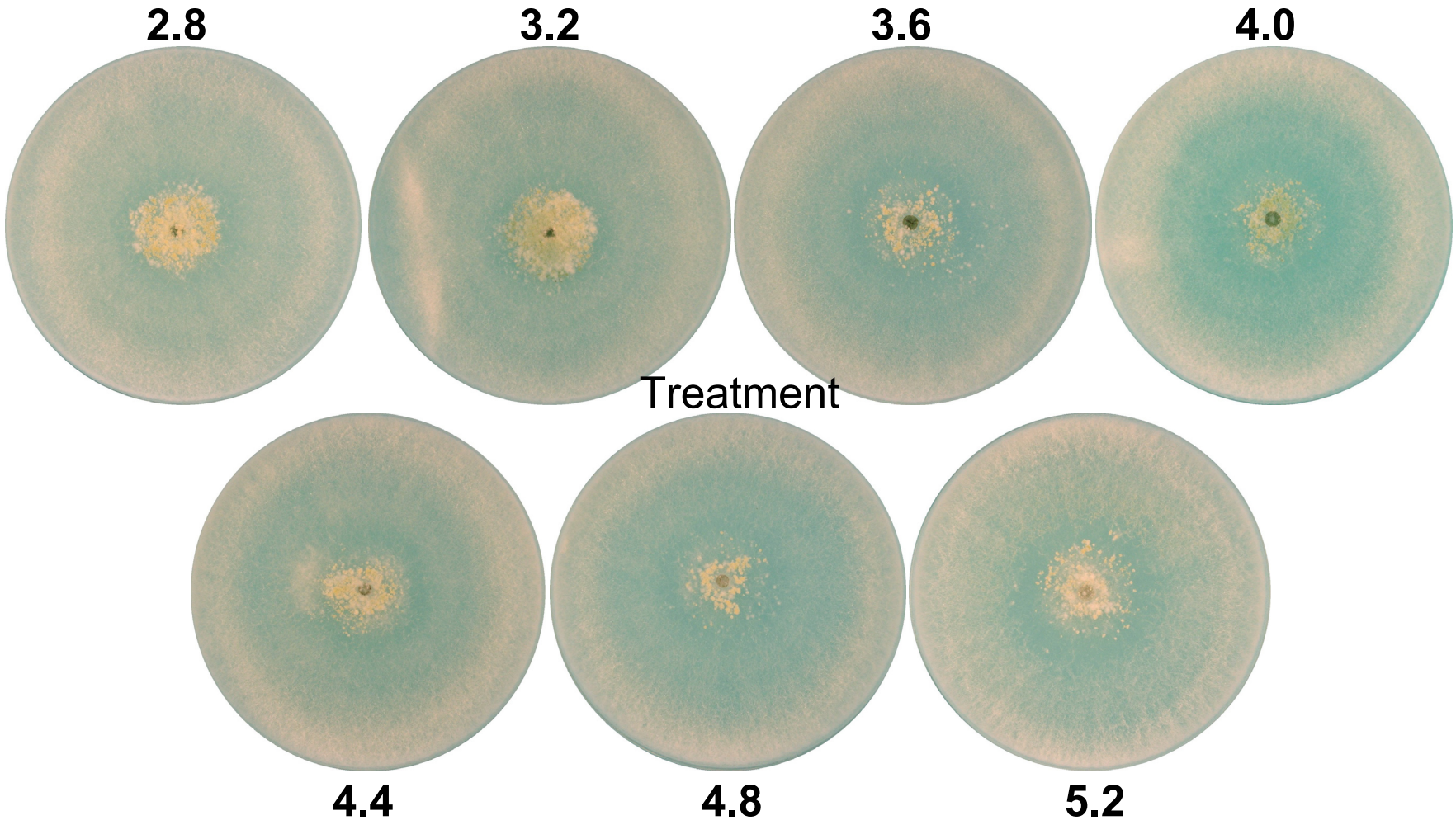


Figure 2.18B. *Trichoderma hamatum* photoconidiation experiment (treatment plates) on unbuffered, pH adjusted (2.8 to 5.2) PDA. Cultures were grown for 48 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

2.11.2.3 Changes in the Medium pH

In contrast to the buffered PDA experiment, the pH values of the unbuffered agar plates shifted dramatically from the start of the experiment. At the time of light exposure, readings were taken at the centre of the colony, the colony edge and the uncolonised plate edge (Table 2.3). The highest value was always at the centre of the plate, followed by the edge and there was no significant change in the uncolonised agar.

Table 2.3. Agar pH readings on unbuffered, pH adjusted, PDA at the time of photoinduction. A = pH at colony centre. B = pH at colony edge. C = pH of surrounding agar.

	***T. atroviride* LU298**			***T. hamatum* LU592**		
Initial pH	**A**	**B**	**C**	**A**	**B**	**C**
2.8	4.4	3.1	2.8	3.3	3.3	2.8
3.2	4.0	4.0	3.3	4.3	4.2	3.3
3.6	4.4	4.0	3.6	4.5	4.2	3.6
4.0	5.0	4.2	4.0	5.3	5.1	4.2
4.4	5.4	4.7	4.4	6.2	5.7	4.4
4.8	6.2	5.2	4.8	6.2	6.1	4.8
5.2	6.3	5.5	5.2	6.2	6.1	5.2

2.11.3 Effect of Culture Age on Photoconidiation at pH 2.8 in *T. atroviride*

Hyphal cell age and metabolic rate have been proposed as the main factors determining competency to photoconidiate (Chapter 1), whereas experiments in this study have demonstrated that competency to respond to stimuli relates to the metabolic state of the cell, and this varies between isolates. On PDA buffered to pH 2.8, a dense disk of conidia was produced in response to light, and this diminished rapidly as the pH rose, which suggests that photoconidiation in *T. atroviride* is strictly dependent on the ambient pH. The margins of the disk produced at pH 2.8 correlated with the position of the colony margin at the time of light exposure. If photoconidiation is strictly low pH-dependent, then as the colony size increases on PDA buffered to pH 2.8, so too should the diameter of the photoconidiation disk. To test this hypothesis, *T. atroviride* was grown on PDA buffered to pH 2.8 in large plates and exposed to light at 24, 48, 72, 96, 120 or 144 h and then photographed 72 h later. Plates were inoculated with either a conidial suspension or a mycelial plug.

2.11.3.1 Conidial Inoculum

In the first experiment, a conidial suspension was used to inoculate control and light treatment plates. The control plates were incubated for the same length of time as each treatment plate and thus represented 4, 5, 6, 7, 8 and 9 d growth in total darkness. After 4 d growth, sparse conidiation was observed surrounding the centre of the control plate (Figure 2.19A). As the plates aged, conidiation increased outwards from the centre and after 8 d green pigmentation was observed in the centre of the cluster. The zone of conidiation extended in size to approximately 45 mm.

A disk was produced in response to light on the treatment plates and this increased in size as predicted, however as the cultures aged, conidiation became uneven in maturity and eventually the response to light ceased. On the 24 h light treatment plates (4 d old) a small dense cluster of conidia was produced at the inoculation site, which correlated to the colony margin at the time of light exposure and this was in addition to conidia clustered around the centre (Figure 2.19B). A green pigmented disk of conidia was observed on the 48 h light treatment plates (5 d old), identical to that observed in 2.11.1.1. On the 72 h light treatment plates (6 d old) a clearly defined ring and thicker diffuse inner ring of pigmented conidia was observed within a conidial disk. The outer ring correlated with the colony margin at the time of light exposure. Intense conidiation was also observed in the centre of the disk. On the 96 h treatment plates (7 d old) a ring of green conidia was observed which also correlated with the colony margin at the time of light exposure, however the response was no longer disk-like. Immature conidia were observed extending approximately 30 mm inside the ring, followed by a clear zone in which hyphae and a few white tufts of conidia were visible. Similar to 72 h treatment plates, a second ring was observed behind the colony front, though this was barely discernible. At the centre of the plate a cluster of colourless to green pigmented conidia were observed and this was the same diameter as the immature conidia observed on the 7 d old control plate. At the time of light exposure on the 120 h treatment plates (8 d old) the colony had reached the edge of the plate. Mature green conidia were observed at the edge of the agar and in the centre a green pigmented cluster which resembled that on the respective control plate was visible. Plates exposed to light at 144 h (9 d old) were indistinguishable from their controls. The pH of the agar did not alter significantly during the experiment.

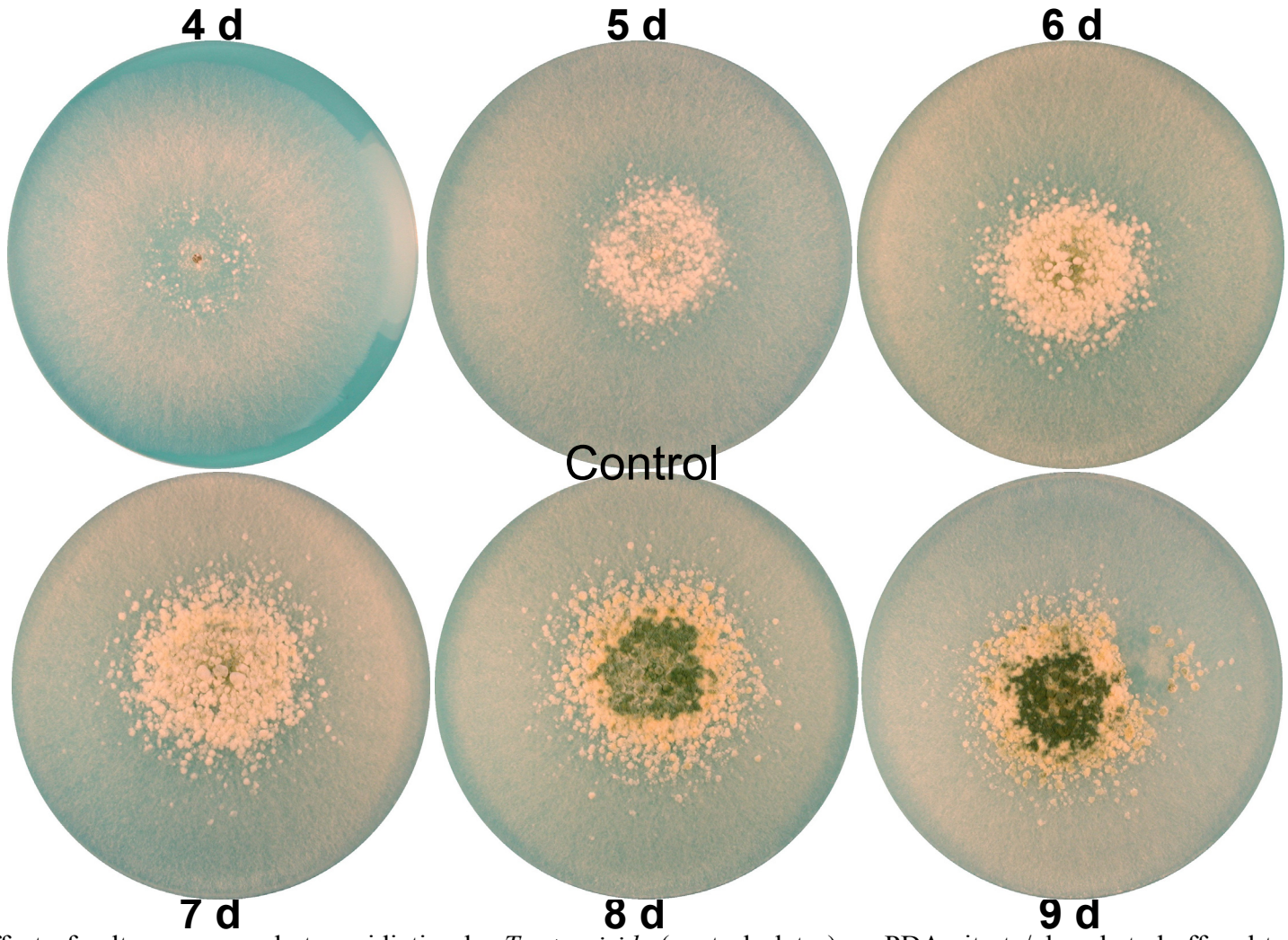


Figure 2.19A. Effect of culture age on photoconidiation by *T. atroviride* (control plates) on PDA citrate/phosphate buffered to pH 2.8. Plates were inoculated with a conidial suspension. Cultures were grown in total darkness for equivalent length of time as the treatment plates. The age of the plates is indicated on the figure in days (d).

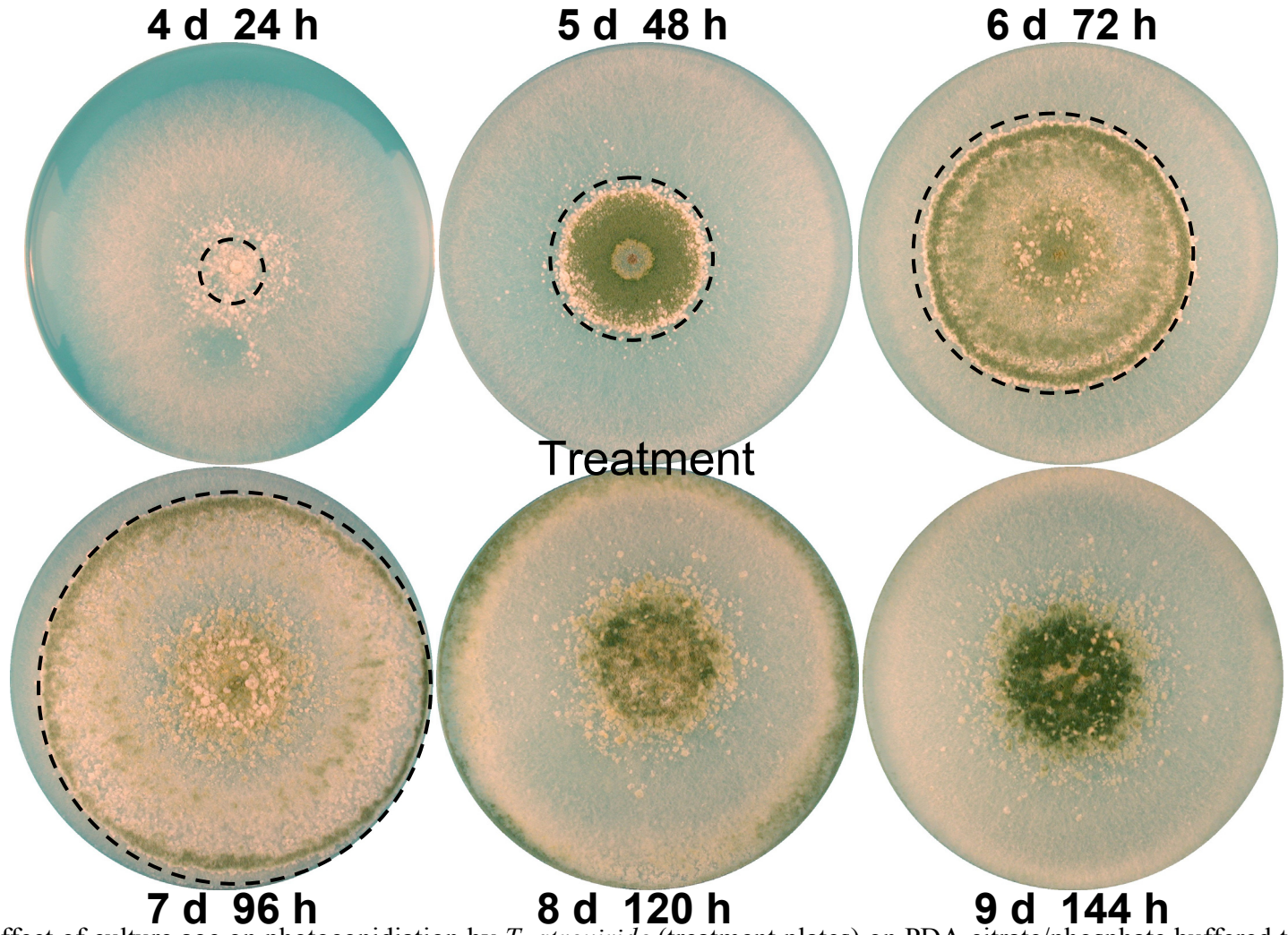


Figure 2.19B. Effect of culture age on photoconidiation by *T. atroviride* (treatment plates) on PDA citrate/phosphate buffered to pH 2.8. Plates were inoculated with a conidial suspension, grown for 1, 2, 3, 4, 5 or 6 d in total darkness, then photoinduced and grown a further 72 h in total darkness. The age of the plates is indicated on the figure in days (d). The time of light exposure is indicated on the figure in hours (h). The dashed black circle represents the colony margins at the time of light exposure.

2.11.3.2 Mycelial Inoculum

In a similar fashion to 2.11.3.1, immature conidiation was observed on the 4 d old control plates (Figure 2.20A). As the plates aged, however, conidiation did not increase in density. Conidia were yellow-green in pigment and from the 7 d old plates onwards the central zone of conidiation was barely visible in the dense mycelial growth. At 8 and 9 d, large white tufts of hyphae were observed and if allowed to incubate longer, these formed conidia.

In contrast to the controls, the mycelial plug inoculum treatment plates closely resembled the conidial suspension plates, though they were more advanced. On the 24 h treatment plates (4 d old) a disk of profuse conidiation was produced (Figure 2.20B) and this was identical to the disk produced on the 48 h treatment plates in 2.11.3.1 (Figure 19B). The mycelial plug 48 h treatment plates (5 d old) closely resembled the conidial inoculum 72 h treatment plates (6 d old), except the outer ring was thicker than the inner ring and sparse immature to yellow-green conidiation was observed at the centre of the plates similar to that on the respective control. Similarly, the 72 h mycelial; plug treatment plates closely resembled the 96 h conidial inoculum treatment plates except the inner diffuse ring was absent. The central zone of conidiation was sparse and resembled the control plates, though more mature in pigmentation. On the 96 h mycelial plug treatment plates (7 d old) conidiation was observed at the edge of the plates and in the centre the zone of conidiation was more intense compared with the respective control and green pigmentation of conidia was observed. In contrast, conidiation at the centres of the 120 h (8 d old) and 144 h (9 d old) treatment plates was sparse and resembled that observed on the control plates. Conidiation was also observed at the edge on both these treatment plates.

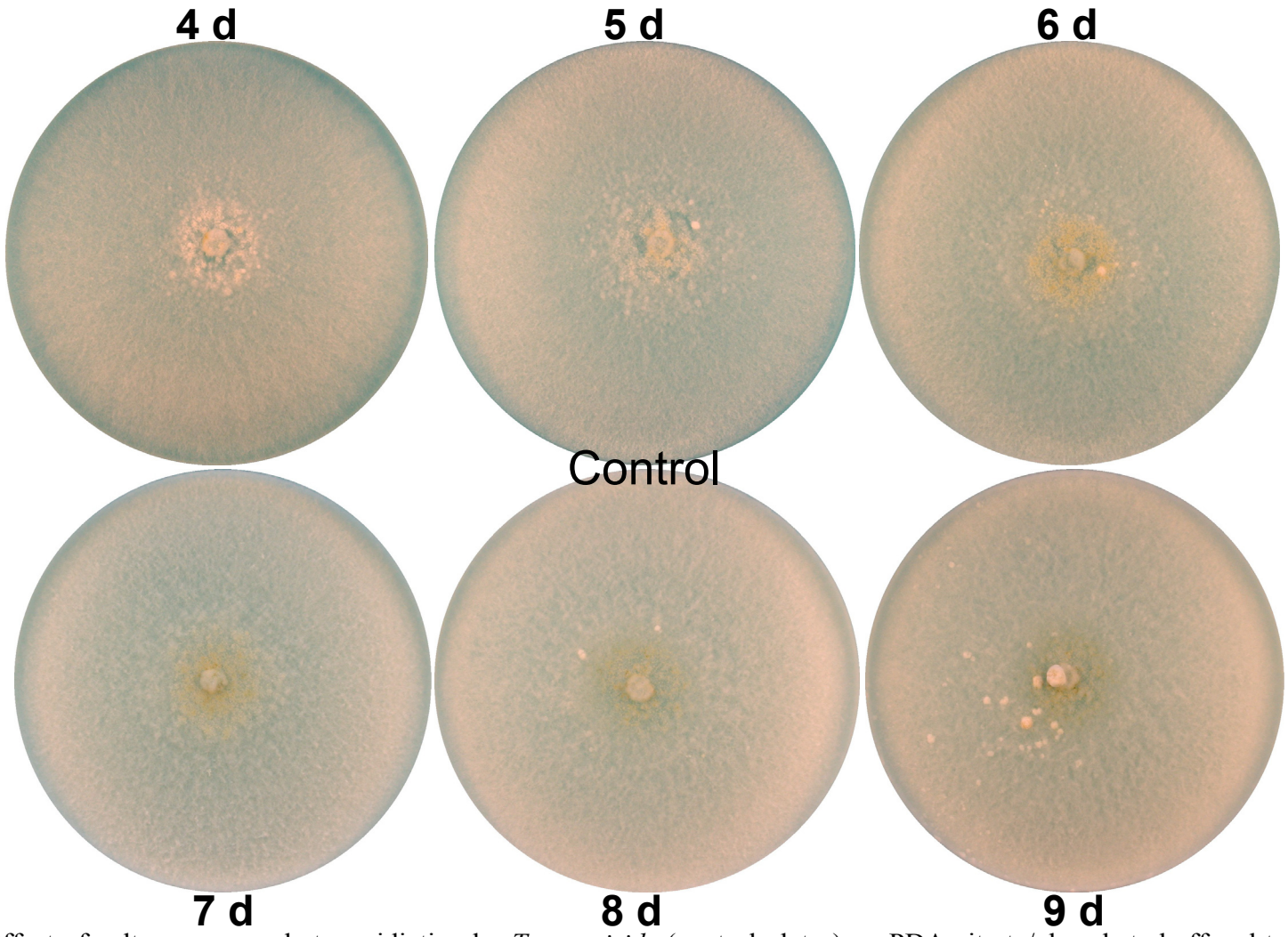


Figure 2.20A. Effect of culture age on photoconidiation by *T. atroviride* (control plates) on PDA citrate/phosphate buffered to pH 2.8. Plates were inoculated with a mycelial plug. Cultures were grown in total darkness for equivalent length of time as the treatment plates. The age of the plates is indicated on the figure in days (d).

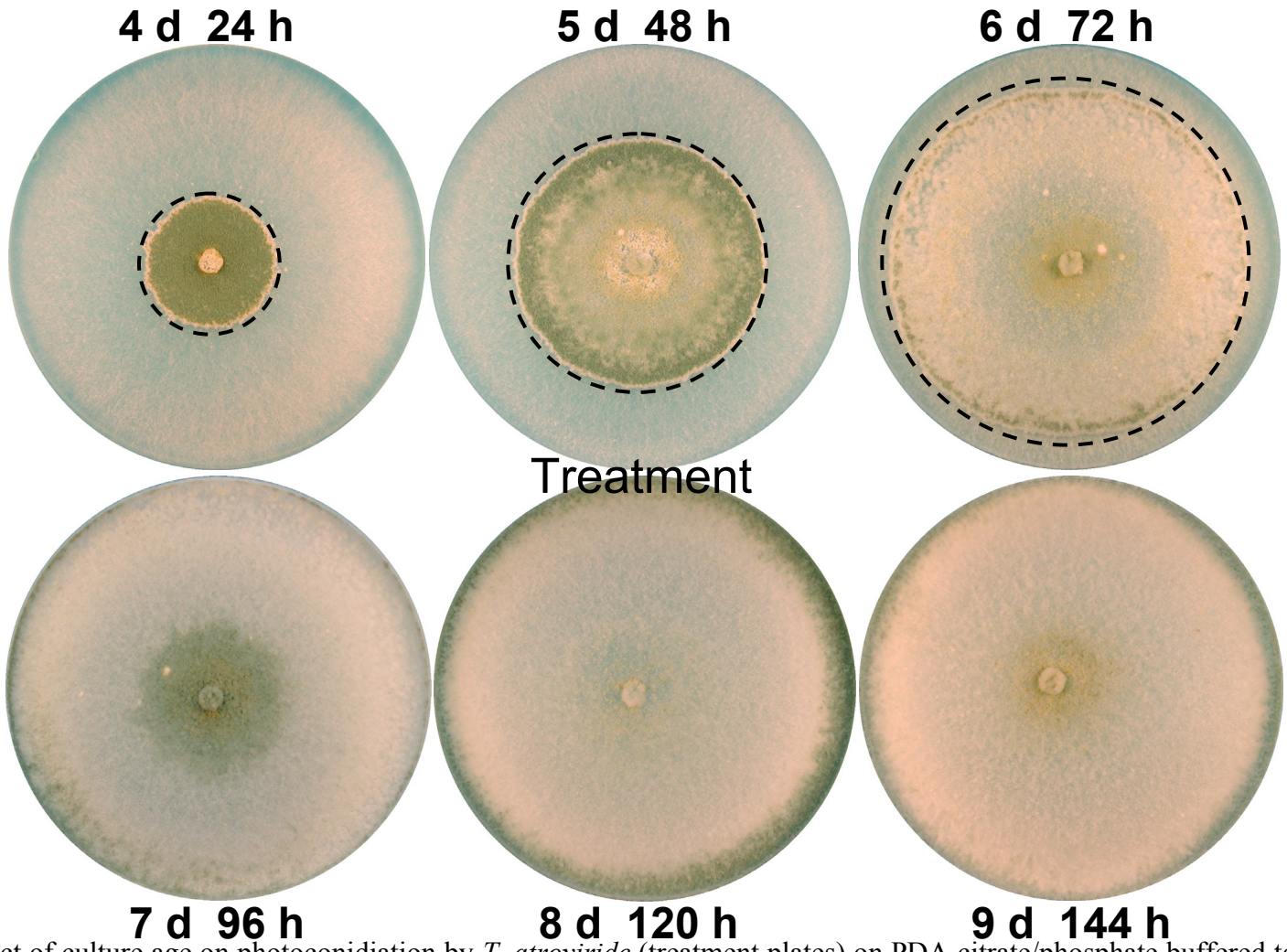


Figure 2.20B. Effect of culture age on photoconidiation by *T. atroviride* (treatment plates) on PDA citrate/phosphate buffered to pH 2.8. Plates were inoculated with a mycelial plug, grown for 1, 2, 3, 4, 5 or 6 d in total darkness, then photoinduced and grown a further 72 h in total darkness. The age of the plates is indicated on the figure in days (d). The time of light exposure is indicated on the figure in hours (h). The dashed black circle represents the colony margins at the time of light exposure.

2.11.4 Effect of Culture Age on Injury-induced Conidiation at pH 2.8 in *T. atroviride*

The effect of increasing culture age was also investigated in relation to injury-induced conidiation. As observed in 2.11.3.1, conidiation in the dark increased as the plates aged, however unlike photoconidiation, conidiation in response to injury also intensified over time (Figures 2.21A & B). No injury response was observed on plates injured at 24 h and although conidiation was more mature on the 48 h treatment plates in comparison with the controls, a clear injury response was not observed until the 72 h treatment plates (Figure 2.21B). Conidiation was observed evenly along the injury sites on cultures injured at 72 h; this was immature to green in pigment and mainly immature at the perimeter. A similar pattern was present on plates injured at 96 h. At 120 h the colony had reached the edge of the plate. Injury-induced conidiation was observed along all the injury sites and at the plate edge conidial pigmentation was more mature than at the centre. Conidiation intensified on colonies injured at 144 h. Conidiation was also observed in the centre of the treatment plates and this was indistinguishable from the controls.

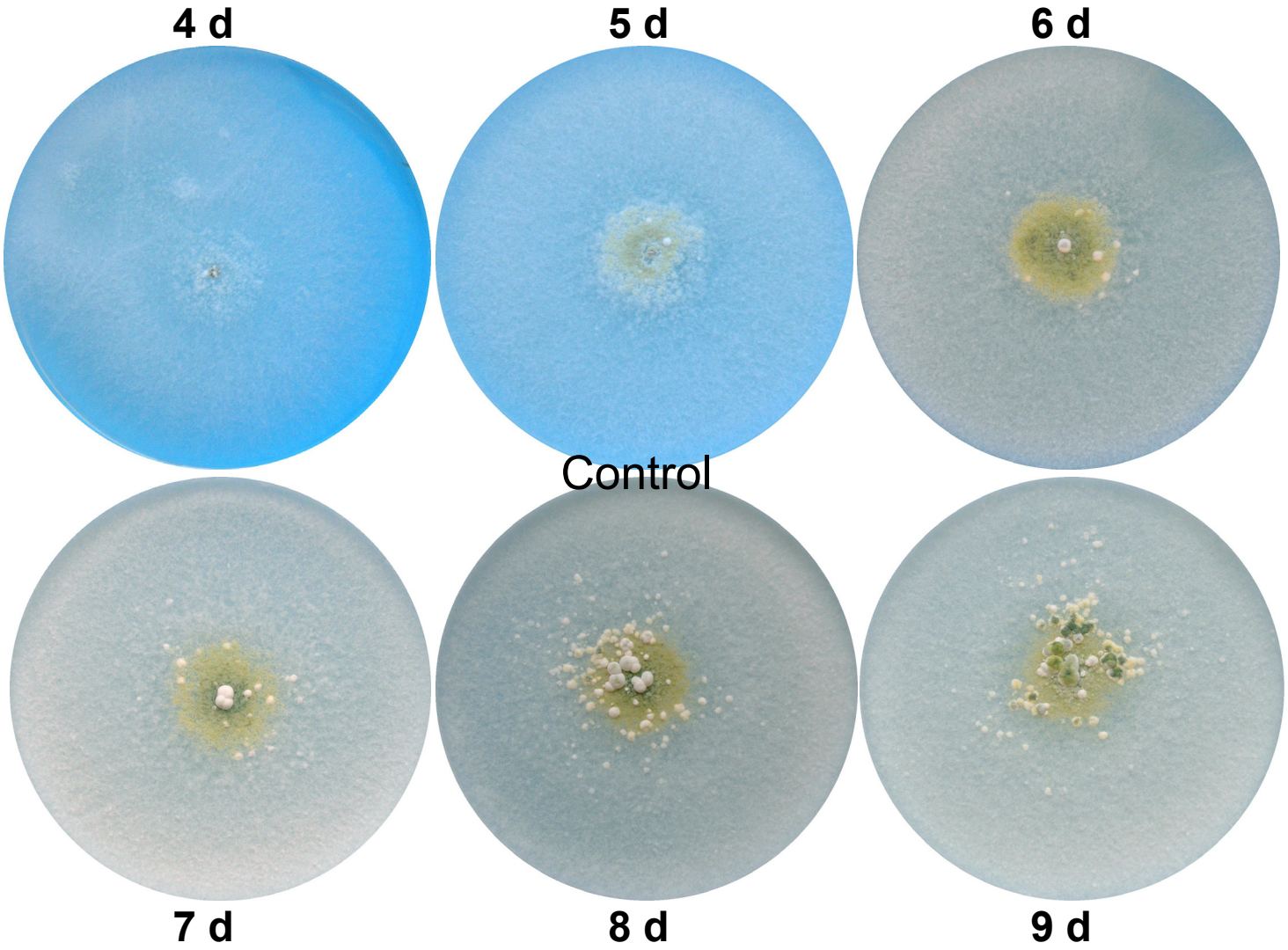


Figure 2.21A. Effect of culture age on injury-induced conidiation by *T. atroviride* (control plates) on PDA citrate/phosphate buffered to pH 2.8. Plates were inoculated with a conidial suspension. Cultures were grown in total darkness for equivalent length of time as the treatment plates. The age of the plates is indicated on the figure in days (d).

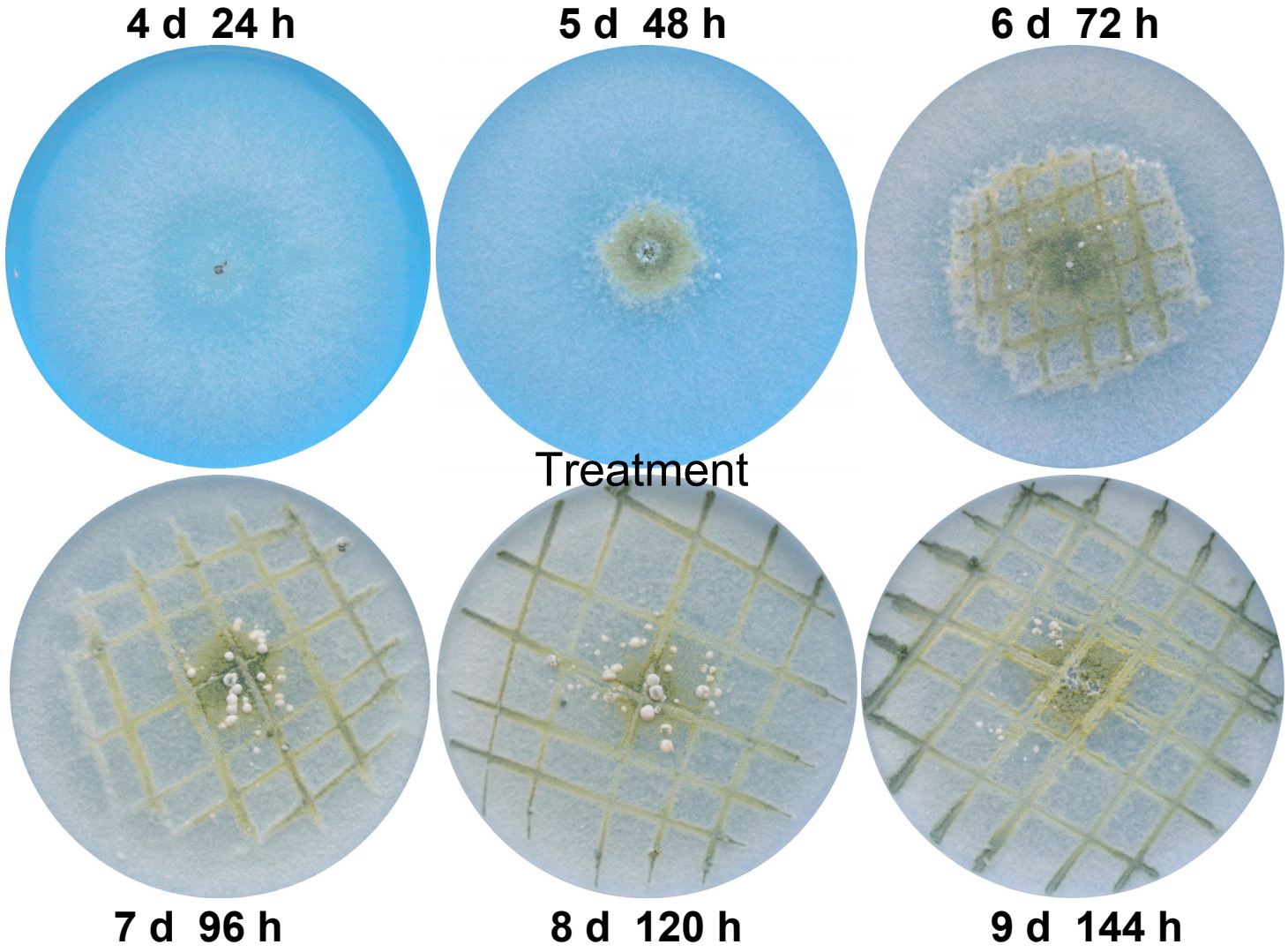


Figure 2.21B. Effect of culture age on injury-induced conidiation by *T. atroviride* (treatment plates) on PDA citrate/phosphate buffered to pH 2.8. Plates were inoculated with a conidial suspension, grown for 1, 2, 3, 4, 5 or 6 d in total darkness, then injured with a scalpel and grown a further 72 h in total darkness. The age of the plates is indicated on the figure in days (d). The time of injury is indicated on the figure in hours (h).

2.11.5 Light Response of *T. harzianum* at pH 2.8 to 5.2

2.11.5.1 pH-buffered PDA with Conidial Inoculum

In the first experiment (2.11.1), the influence of ambient pH on photoconidiation in *T. atroviride* and *T. hamatum* was investigated using pH-buffered PDA (2.8 to 5.2). In contrast to *T. atroviride* and *T. hamatum*, conidiation in *T. harzianum* was observed in all control and treatment plates. Unexpectedly, three distinct colony morphologies were observed and these appeared to be pH-dependent. At pH 2.8, immature conidiation was observed on the 5 d old control plates and this increased in density and maturity to an optimum at pH 3.6 (Figure 2.22A). From pH 4.0 to 4.4, a distinct change in colony morphology was observed. Colony growth was constrained compared with growth at higher or lower pH values and the colony margins were undulating, which resembled the stress morphology described previously for *T. atroviride* at pH 5.6 or greater (2.7.5). Unlike *T. atroviride*, conidiation was observed on these plates. At pH 4.4, the hyphae at the colony margin had a feather-like appearance (Figure 2.22A.II). Dramatic changes in morphology were observed at pH 4.8 and 5.2. A yellow pigment was secreted into the medium at both pH values and the hyphal morphology was spoke-like as observed on buffered (pH 5.4) glutamine and urea-amended MM agar (2.7.1.1). Immature conidiation was observed at pH 4.8, though at pH 5.2 it was very sparse.

A conidial response to light was observed from pH 2.8 to 4.0 only and at higher pH values there was no discernible difference between control and treatment plates. At pH 2.8, a small ring-like zone of colourless to green pigmented conidia was observed at the centre of the plate which correlated with the position of the colony margin at the time of light exposure (Figure 2.22B). A second ring of colourless to green conidia was observed beyond the central cluster. Conidiation also occurred on the pH 2.8 control plates at this position on the plate, though on the controls it was sparser, immature and more diffuse. Photoconidiation in the centre increased in density and maturity on the pH 3.2 treatment plates. This also was surrounded by a second region of colourless to green conidia, which appeared ring-like but more diffuse than on the pH 2.8 treatment plates. At pH 3.6, conidiation in response to light was similar in maturity and density to pH 3.2, however unlike that observed at pH 2.8 and 3.2, the edges of the ring diffused into the background conidiation, which was observed in the controls. The margins of the colony were undulating like the morphology observed on the pH 4.0 and 4.4 control plates. A thin ring of green conidia was produced in response to light at pH 4.0, however the hyphae resembled the feather-like appearance of the pH 4.4 control plates.

No discernible difference was observed between the pH 4.4, 4.8 and 5.2 treatment plates and their respective controls, however the colonies were less than 10 mm in diameter at the time of light exposure. The pH of the agar did not alter significantly during the experiment.

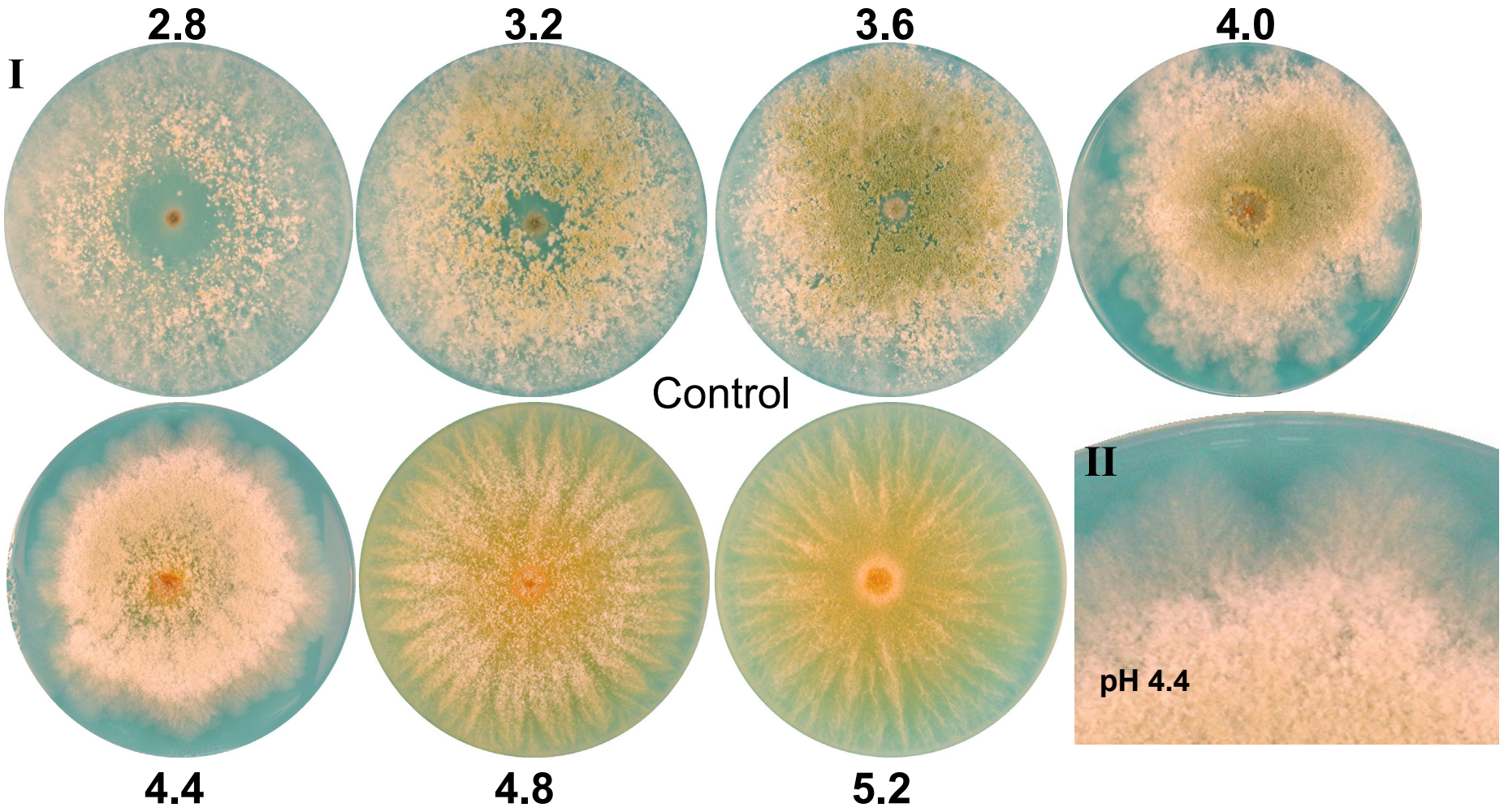


Figure 2.22A. I. *Trichoderma harzianum* photoconidiation experiment (control plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Plates were inoculated with a conidial suspension. Cultures were grown for 5 d in total darkness. **II.** Enlargement of the pH 4.4 colony margin.

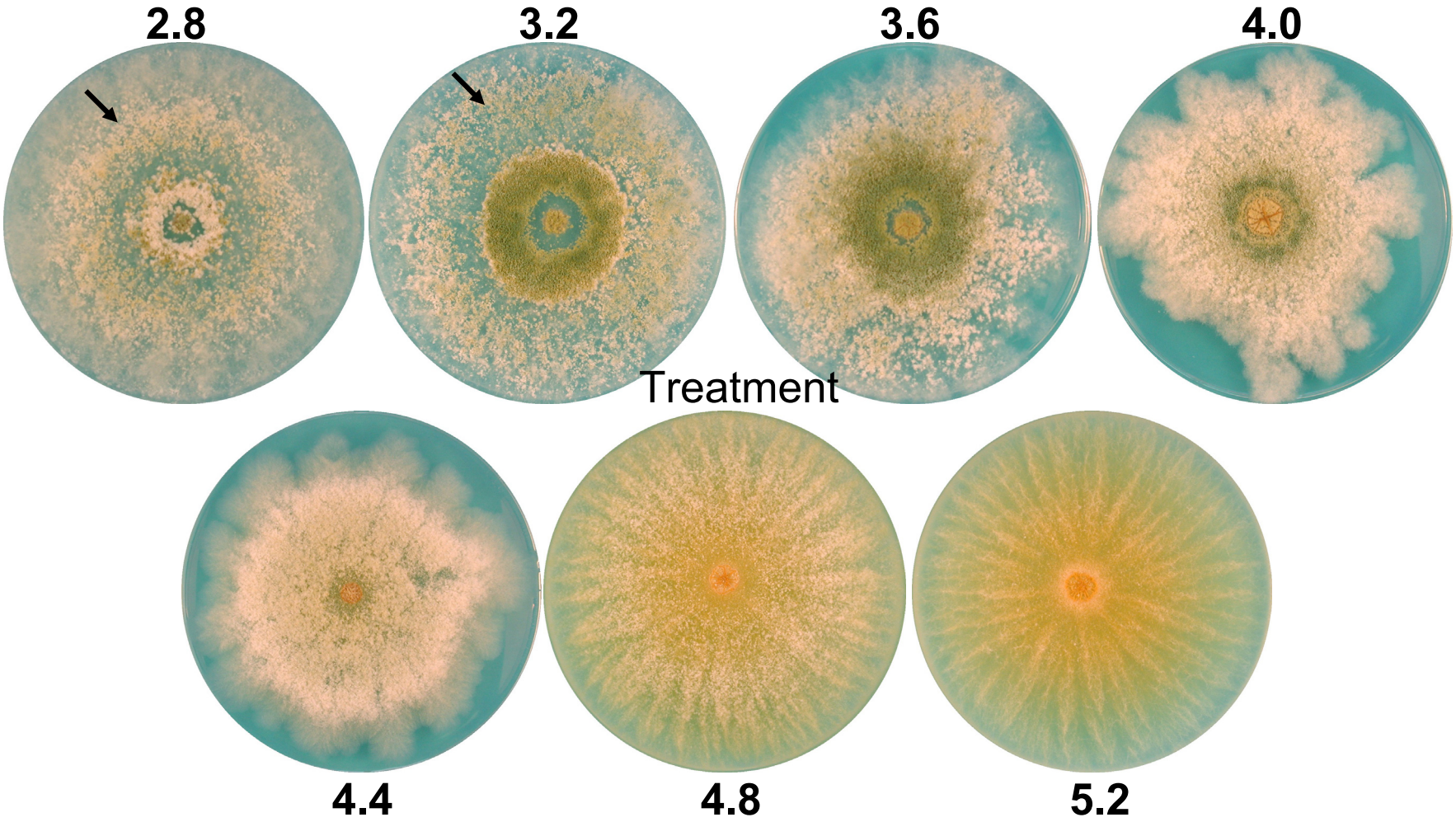


Figure 2.22B. *Trichoderma harzianum* photoconidiation experiment (treatment plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Plates were inoculated with a conidial suspension. Cultures were grown for 48 h in total darkness, then photoinduced and grown a further 72 h in total darkness. The arrows point to conidial rings produced from hyphae not formed at the time of light exposure.

2.11.5.2 pH-buffered PDA with Mycelial Inoculum

The mycelial inoculum controls closely resembled the conidial inoculum control plates (2.11.5.1), except that conidiation was more intense and pigmentation was more advanced (Figure 2.23A). Conidiation occurred on all plates and, as observed on the conidial inoculum control plates, three distinct colony morphologies were observed. The stress morphology was clearly evident from pH 4.0 to 4.8 and this was more exaggerated than on the conidial inoculum controls. The yellow pigment was present from pH 48 to 5.2, however the spoke-like pattern was much less pronounced. Interestingly, at pH 2.8 and 3.2 a ring of green-yellow conidia was observed in the centre of the plates surrounding the mycelial plug, whereas on the conidial inoculum controls mycelial growth was sparse and conidiation absent in the centre.

In response to light, a disk of profuse conidiation was produced on the mycelial inoculum treatment plates from pH 2.8 to 4.4 and this correlated with the colony margins at the time of light exposure (Figure 2.23B). This differed from the conidial inoculum experiment where a conidial response to light was observed from pH 2.8 to 4.0 only and likely reflects the larger colony size at the time of light exposure. From pH 3.6 to 4.0 the margins of the disk were irregular, whereas the stress morphology in terms of colony margin undulation was clearly observed from pH 4.0 to 4.4. No clear photoconidiation response was observed from pH 4.8 to 5.2, however in comparison with the mycelial inoculum controls conidiation appeared reduced and was not evenly distributed. In the centre there was a cluster of immature to yellow conidia followed by a sparser ring and then a denser ring of conidia and these lay in a region of dense mycelium. No part of the conidiation pattern correlated with the colony margin at the time of light exposure. The yellow pigment was also present from pH 4.8 to 5.2, although the spoke-like pattern was barely discernible. The pH of the agar did not alter significantly during the experiment.

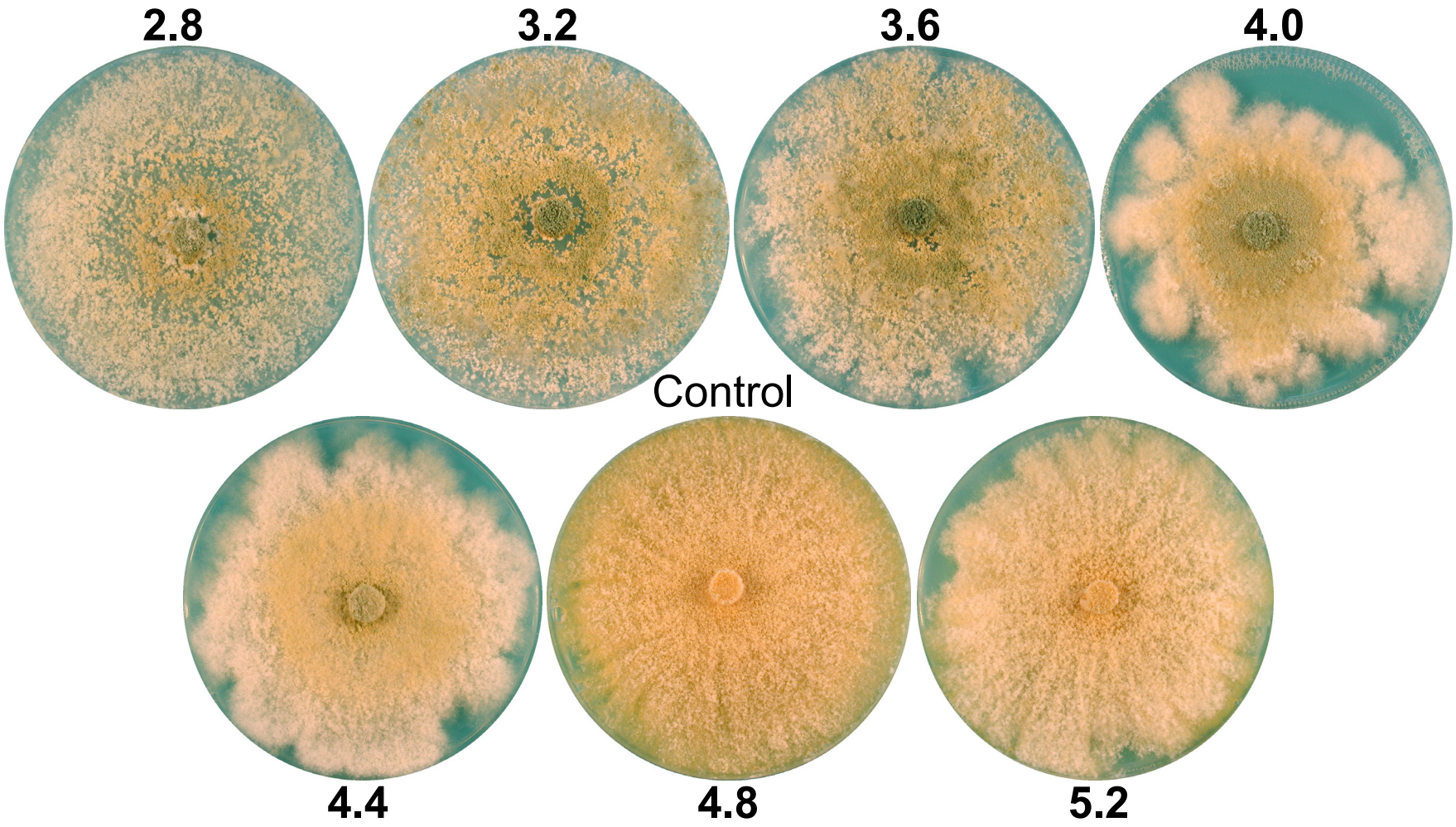


Figure 2.23A. *Trichoderma harzianum* photoconidiation experiment (control plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Plates were inoculated with a mycelial plug and grown for 5 d in total darkness.

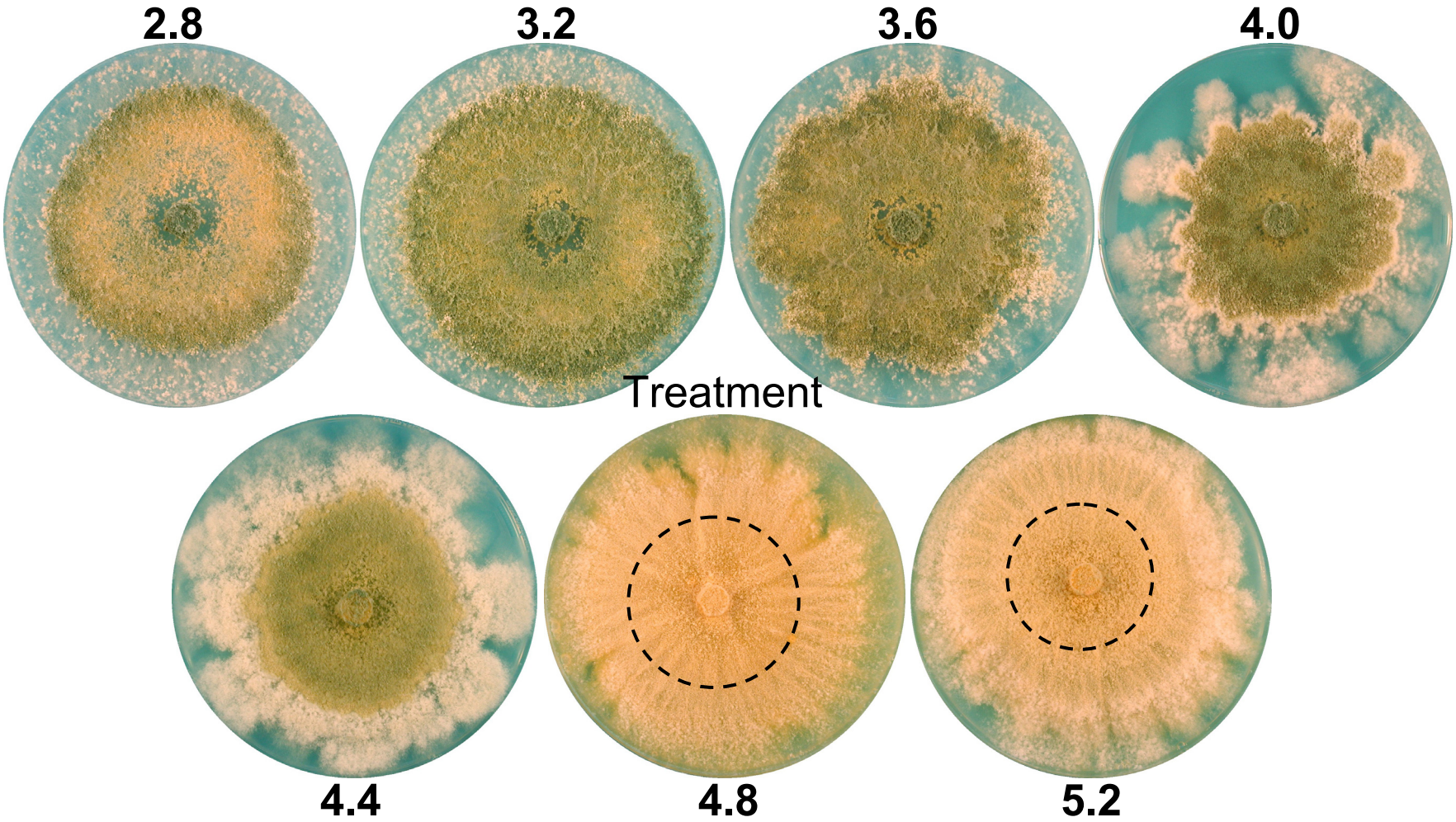


Figure 2.23B. *Trichoderma harzianum* photoconidiation experiment (treatment plates) on citrate/phosphate buffered (2.8 to 5.2) PDA. Plates were inoculated with a mycelial plug. Cultures were grown for 48 h in total darkness, then photoinduced and grown a further 72 h in total darkness. The dashed black circle represents the pH 4.8 and 5.2 colony margins at the time of light exposure.

2.11.5.3 Unbuffered PDA with Mycelial Inoculum

Mycelial plugs were also used as inoculum in a photoconidiation experiment using unbuffered PDA which had been acidified prior to sterilisation. Colony morphology altered as the pH rose, however the changes were not as distinct compared with the buffered experiment. The stress morphology was only discernible at pH 4.0 (Figure 2.24A) and this was less pronounced than on the buffered control plates. At pH 4.8 the spoke-like morphology and yellow pigment observed on the respective buffered control were absent and conidiation occurred evenly across the plate. Conidial pigmentation ranged from green to yellow-green and no colourless conidia were observed on the plates. The spoke-wheel morphology was also absent at pH 5.2, however some yellow pigment was observed in the medium and conidiation was less dense and yellow-green in pigment.

Conidiation in response to light occurred at all pH values on the unbuffered PDA and this differed from the buffered experiment, in which a conidial response was observed from pH 2.8 to 4.4 only. Conidiation occurred in a disk, which correlated with the colony margin at the time of light exposure, and as the pH rose this disk became more ring-like in appearance. From pH 2.8 to 4.4 the disks were predominantly green in pigment and the margins of the disks were irregular (Figure 2.24B), similar to the photoconidiation disks produced from pH 3.6 to 4.0 on buffered PDA (2.11.5.2). The conidial disks produced from pH 4.8 to 5.2 had a regular margin unlike the previous pH values. At pH 4.8, pigmentation was yellow-green in the centre and green around the edges in a ring-like fashion. Though not apparent on the control plates there appeared to be some arrangement of the conidia in a spoke-like morphology. The appearance of these plates differed significantly from the buffered pH 4.8 plates on which no conidial changes between control and treatment plates were observed and a yellow pigment was excreted into the medium. At pH 5.2, pigmentation was predominantly yellow-green and at the edge of the disk a clearly defined ring of green conidia was produced. As with pH 4.8, this morphology differed significantly from the buffered pH 5.2 plates on which no conidial changes were observed between the control and treatment plates. As observed on the control plates, the spoke-wheel morphology was absent, however some yellow pigmentation was observed in the medium. There was no significant alteration of the pH of the agar during the experiment.

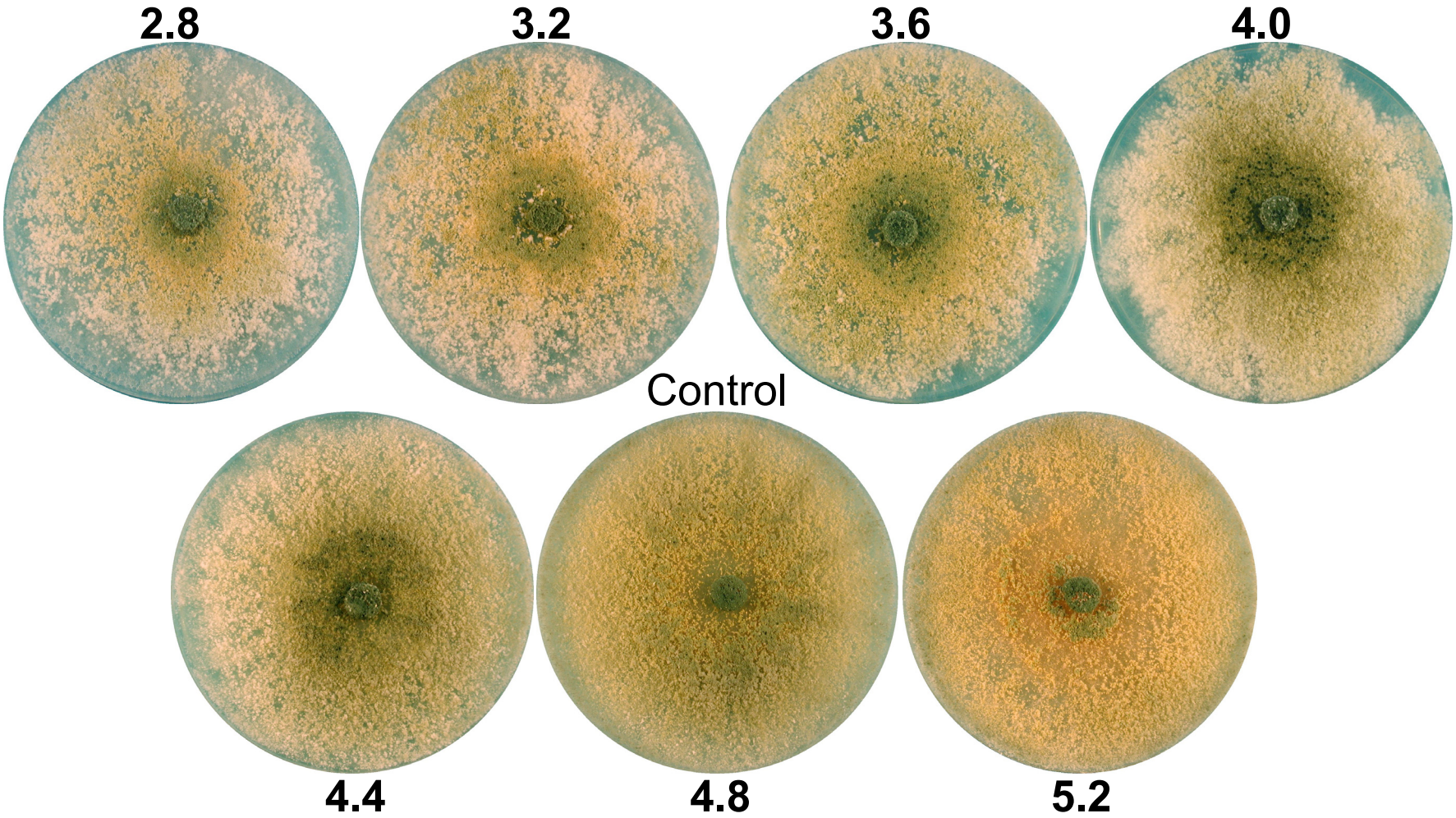


Figure 2.24A. *Trichoderma harzianum* photoconidiation experiment (control plates) on unbuffered, pH adjusted (pH2.8 – 5.2) PDA. Plates were inoculated with mycelial plugs. Cultures were grown for 5 d in total darkness.

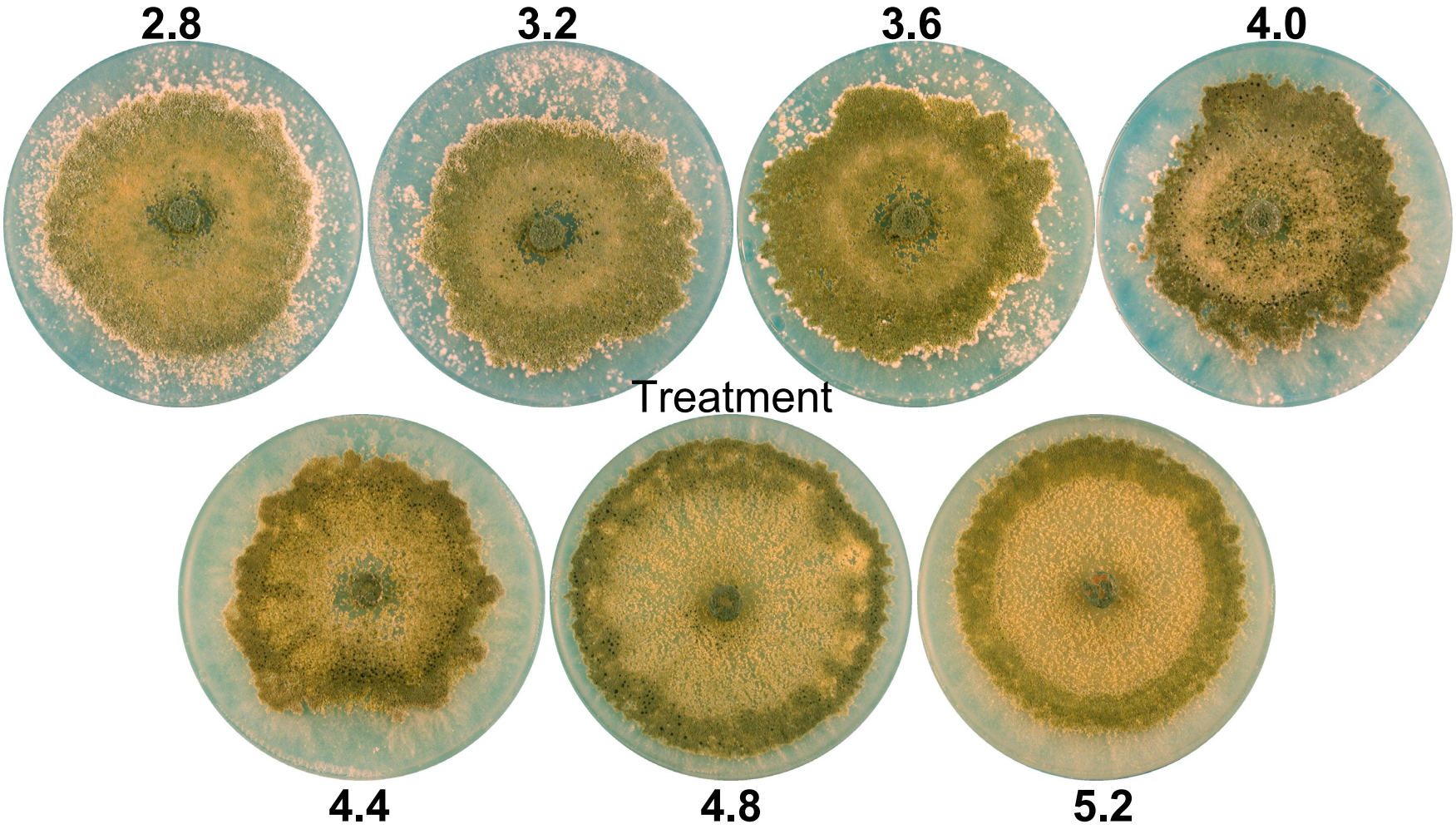


Figure 2.24B. *Trichoderma harzianum* photoconidiation experiment (treatment plates) on unbuffered, pH adjusted (pH2.8 – 5.2) PDA. Plates were inoculated with mycelial plugs. Cultures were grown for 48 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

2.12 Discussion

2.12.1 Low pH Promotes Conidiation in *Trichoderma atroviride*

In *T. atroviride*, on pH-buffered PDA, photoconidiation was restricted to low pH. No conidiation occurred above pH 4.0, and from pH 2.8 to 3.6 a disk of conidia was produced in response to light. The production of a disk at low pH clearly demonstrates that, as with both *T. harzianum* and *T. asperellum* (Sections A and B), competency to conidiate in response to a single light burst is not constrained to the perimeter in *T. atroviride*, rather it is dependent on the metabolic state of the cell as determined by the environment. As the pH lowered, conidiation intensified and the disk increased in maturity from the centre outwards, which suggests that on buffered PDA, competency to photoconidiate increases as the pH decreases. Conidiation in the absence of stimuli was also low pH-dependent. Nutritional studies have previously demonstrated that both conidiation and growth are favoured by low pH (section 1.4.1), however to the best of our knowledge this is the first time photoconidiation and conidiation in the dark have been demonstrated to be low pH-dependent in *Trichoderma*.

In contrast to the buffered experiments, photoconidiation occurred in *T. atroviride* at all pH values tested on unbuffered medium, though conidiation was most intense at pH 3.6. As the initial pH of the medium rose, conidiation decreased in intensity and became scarce at the centre of the colony. Conidiation in the dark did not occur above pH 4.0, which suggests that conidiation in the dark is strictly low pH-dependent. In Section A, this isolate photoconidiated on PDA in a ring which appeared partially filled in (Figure 2.2C). The pH of the agar was not determined prior to inoculation, but based on the readings taken during preparation of agar in this section and manufacturer's specifications it was assumed to be between pH 5.6 and 5.8. Together this suggests that on unbuffered medium, competency to photoconidiate in this *T. atroviride* isolate decreases and became constrained to the perimeter as the initial pH value rises.

On buffered PDA, photoconidiation by *T. atroviride* was low pH-dependent, whereas on unbuffered PDA it occurred at all pH values tested. Buffered medium is routinely used to investigate the effect(s) of ambient pH on various physiological and biochemical parameters due to the ability of fungi to modify the ambient pH and/or adapt to the environment by altering intracellular pH. Indeed, in Section B, on unbuffered MM with glutamine, the agar under the *T. atroviride* growing front was acidified relative to the initial pH. The low pH region corresponded with both light and

injury-induced conidiation, which suggests that acidification of the local environment promotes photoconidiation. At the time of light exposure, changes in the pH of the agar were observed on all plates, however alkalisation rather than the predicted acidification occurred. At the time of light exposure, the ambient pH of the agar was higher under the centre of the colony than under the hyphal front, which was slightly higher than the uncolonised agar, thus establishing an ambient pH gradient radiating from the centre outwards. The only exception was when the initial pH was at 3.2. On these plates the ambient pH was at 4.0 under the whole colony at the time of light exposure. Interestingly, pH 4.0 has been demonstrated to be optimal for mycelial growth of *T. aureoviride*, *T. harzianum* and *T. viride* on buffered yeast extract agar and *T. viride* in liquid medium (Kredics *et al.*, 2004; Brian & Hemming, 1950).

Alkalisation of the growing medium has been observed previously in liquid cultures of *T. viride*, *T. harzianum* and *T. hamatum* (Lewis & Papavizas, 1983). In molasses-corn steep liquor or minimal media with secondary nitrogen and carbon sources the ambient pH rose. In contrast, when primary nitrogen and carbon sources were used, acidification of the broth was observed. Similarly, our studies have demonstrated alkalisation of PDA (a complex medium similar to molasses-corn steep liquor) and acidification on MM with primary nitrogen and carbon sources. Alkalisation of the medium is a common occurrence amongst fungal species. Similar to *T. atroviride* LU298, *Metarhizium anisopliae* established a pH gradient on unbuffered complex agar medium, in which the highest value was in the centre of the colony, (St Leger *et al.*, 1999). These authors also observed alkalisation of liquid media by *M. anisopliae*, *Aspergillus fumigatus* and *Neurospora crassa* and this was linked to ammonia production. Filamentous fungi alkalinise the medium through the secretion of ammonia (Prusky & Yakoby, 2003; St Leger *et al*, 1999). The degree to which ammonia secretion alkalinises the medium has been shown to be dependent on the initial pH, the buffering of the environment and the amount and source of nitrogen (Eshel *et al*, 2002; Drori *et al*, 2003). Based on the results presented in this study and observations from the literature it is proposed that on PDA, *T. atroviride* alkalinises the medium by the secretion of ammonia and, further, on MM with primary nitrogen sources only, acidification of the agar under the hyphal front is related to the sole nitrogen source.

In *T. atroviride*, photoconidiation was strictly low pH-dependent on buffered PDA, whereas it occurred at all pH values on unbuffered media and was not linked to

acidification of the ambient pH as hypothesised. One possible explanation for this observation is that conidiation may be dependent on intracellular acidification, which, in turn, is dependent on ambient pH and the buffering state of the medium. Low ambient pH of the growth medium has been demonstrated to result in intracellular acidification in *Aspergillus niger* and *Saccharomyces cerevisiae* (Gradišnik-Grapulin & Legiša, 1997; Caspani *et al.*, 1985). In *T. viride*, intracellular acidification occurs when hyphae are exposed to light (Grešik *et al*., 1991), however it is not known whether this response is constrained in a highly buffered environment. Together these studies suggest a possible mechanism for the promotion of photoconidiation in *T. atroviride* at low pH and the apparent disparity between the buffered and unbuffered experiments: On buffered PDA low ambient pH stimulates intracellular acidification and light-induced acidification is constrained, whereas on the unbuffered medium light stimulates intracellular acidification, which brings the internal pH to below the maximum for photoconidiation. As the initial pH rises and pH gradient increases, conidiation is reduced in intensity and became constrained towards the perimeter as the pH under the centre of the colony approaches neutral. If low intracellular pH promotes conidiation, this suggests that in addition to the ambient pH gradient, *T. atroviride* also establishes an intracellular pH gradient. As the internal pH gradient increases, light-induced acidification is no longer sufficient to reduce the pH level at the centre and conidiation becomes constrained to the perimeter. Conidiation in the dark was not observed at the higher pH values on unbuffered medium, which suggests that in the absence of additional stimuli the intracellular pH likely remains constant with the ambient pH and thus is not low enough to stimulate conidiation. Thus it is proposed that photoconidiation is dependent on a low intracellular pH in *T. atroviride*.

Conidiation in response to injury also required a low pH on buffered media, whereas on unbuffered PDA injury-induced conidiation was clearly observed at higher pH (pH 5.6-5.8) and was more intense at the perimeter (Section B, Figure 2.11B; Section A, Figure 2.3B). If conidiation in response to injury is also dependent on low intracellular pH, as postulated for photoconidiation, then injury-induced conidiation at pH values above pH 4.0 on unbuffered PDA is likely due intracellular acidification.

Intracellular acidification in *T. viride* has been proposed to contribute to the observed rise in cAMP following illumination, which in turn likely promoted conidiation (Grešik *et al.*, 1991; Section 1.5.4.1). Intracellular acidification correlates with a rise in cAMP

in *A. niger* and *S. cerevisiae* (Gradisnik-Grapulin & Legisa, 1997; Caspani *et al.*, 1985). In *T. virens*, cAMP has been shown to be essential for conidiation in the dark and in both *T. viride* and *T. atroviride* it has been shown to strongly promote photoconidiation (Grešik *et al.*, 1988; Casas-Flores *et al.*, 2006; Mukherjee *et al.*, 2007; section 1.5.4). It is likely that intracellular acidification occurs in this *T. atroviride* isolate, that it is associated with a rise in cAMP, and promotes photoconidiation and conidiation in the dark and possibly injury-induced conidiation.

The proposed relationships between intracellular pH and conidiation could be tested by investigating intracellular pH levels across the colony under variable conditions, however direct measurements of intracellular pH are not currently possibly. Accurate measurement of intracellular pH would involve the insertion of special micro-electrodes, however the insertion itself involves exposure to light (Gresik *et al.*, 1991). It may be possible to overcome these limitations by using modern fluorescence marker genes. The DsRed protein has an excitation and emission wavelength within the non-responsive red wavelengths. It may be possible, therefore, to insert micro-electrodes in hyphae of *T. atroviride* transformed with the DsRed gene under safe red lighting.

2.12.2 Low pH Promotion of Conidiation is Differentially Regulated in Response to Nutrient Deprivation

Multiple abiotic factors are known to influence conidiation in *Trichoderma* spp., including the carbon and nitrogen levels and carbon to nitrogen ratio and the ambient pH. Depending on the relative combination of these and other factors, some signalling pathways will predominate over others. PDA buffered to pH 2.8 strongly promoted photoconidiation, conidiation in the dark and injury-induced conidiation, which suggests that under these conditions ambient pH is the main influence determining conidiation. If photoconidiation on PDA buffered to pH 2.8 is related solely to the ambient pH, then as the colony size increases, so too should the disk. To test this theory, *T. atroviride* was grown on pH 2.8 buffered PDA in large Petri dishes and exposed to light at variable intervals. Indeed, the disk did increase in diameter as the colony aged, but it became less intense and uneven in maturity and eventually the colony ceased to conidiate in response to light. Concurrently, conidiation on the control plates increased with time in intensity and maturity from the centre outwards. On plates exposed to light at 96 h or later, conidiation at the centre was virtually indistinguishable from the control plates and photoinduced conidiation was absent at the centre and more intense at the

perimeter. These results clearly demonstrate that promotion of photoconidiation by ambient pH is not absolute and suggests that over time the influence of pH on photoconidiation is negatively cross-regulated by another factor(s), which may promote conidiation in the absence of light.

Conidiation in the absence of light or injury stimuli by *T. atroviride* increased over time, whereas photoconidiation decreased. Nutrient deprivation over time would have stimulated starvation induction of conidiation at the centre of the plates. It is possible that nutrient deprivation negatively regulates photoconidiation. Starvation induction occurred from the centre outwards, whereas on plates exposed to light at 96 and 120 h conidiation in response to light was noticeably absent in the centre. If deprivation of key metabolites negatively regulates photoconidiation, then the distribution of conidia produced in response to light suggests compartmentalisation of primary metabolites towards the perimeter. In Section B, it was postulated that in *T. asperellum*, primary nitrogen metabolites were redistributed to the colony perimeter when primary nitrogen in the environment was limiting thus stimulating a ring of conidia in response to light and when primary nitrogen was in abundance, photoconidiation occurred in a disk. A similar mechanism may be occurring over time at low pH in *T. atroviride* in response to nutrient deprivation: As nutrients become depleted, primary metabolites are translocated towards the colony perimeter and photoconidiation does not occur in the centre of the colony.

The nature of the limiting factor(s) which constrained photoconidiation is not known, but based on results in this study to date, nitrogen is a possible candidate. In Section B, primary nitrogen sources were demonstrated to promote photoconidiation in *T. asperellum* and in *T. harzianum*. No direct link between the nitrogen status of the medium and photoconidiation in *T. atroviride* was observed, however based on results from this section, it is highly likely that in the nitrogen experiments, photoconidiation was at least partially constrained by the ambient pH. In *Yarrowia lipolytica* it has been demonstrated that the ambient pH regulates morphogenesis through modulating the availability of organic sources of nitrogen (Szabo & Stofanikova, 2002). Low ambient pH favours the uptake of primary sources of nitrogen such as glutamine (Chalot & Brun, 1998; Horák, 1997). If this also occurs in *T. atroviride*, then on PDA buffered to pH 2.8 primary nitrogen uptake would be favoured. Preferential uptake of primary nitrogen over time would result in rapid depletion and thus primary nitrogen would

become limited in the media. Nitrogen starvation has been previously demonstrated to induce conidiation in the dark in *T. atroviride* (Casas-Flores *et al.*, 2004). It is possible that nitrogen deprivation over time promotes conidiation in the dark and negatively regulates photoconidiation in *T. atroviride* LU298.

As photoconidiation became constrained to the perimeter over time a clearly defined outer ring of mature conidia was observed. Unexpectedly, a second ring of conidia was observed on plates exposed to light at 72 and 96 h. It was postulated in Section B that constraint of photoconidiation towards the perimeter in this experiment is due to translocation of primary metabolites to the hyphal front. If this theory is true then the presence of a second region of mature conidiation suggests that compartmentalisation of primary metabolites under conditions of nutrient limitation is not directed solely towards the hyphal front and other zones of accumulation may occur within the colony. It is also possible that the appearance of two rings may be associated with an endogenous rhythmic sensitivity to light. A subtle endogenous rhythmic change in light sensitivity, as measured by conidiation, has been observed previously in *T. atroviride* on unbuffered complex media at approximately pH 5.6 (Deitzer *et al.*, 1988). The authors did not determine the mechanism behind the rhythmic sensitivity, however endogenous metabolic rhythms which affect morphogenesis have been observed before in fungi (Ramsdale, 1999). In *N. crassa*, the *nit-3* gene is expressed in a rhythmic fashion due to the kinetics of a feedback cycle, which results in the rhythmic accumulation of primary nitrogen in the cell (Christensen *et al.*, 2004). An excess of primary nitrogen inhibits *nit-3* expression and therefore the rhythm. A possible mechanism involving the *nit-3* rhythm may proceed as follows: On PDA buffered to pH 2.8, primary nitrogen uptake by *T. atroviride* is favoured, which inhibits the *nit-3* rhythm. As nitrogen becomes depleted, *nit-3* expression is induced, resulting in the appearance of the *nit-3* rhythm and compartmentalisation of nitrogen reserves. It is also possible that rhythmic light sensitivity observed in *T. atroviride* by Deitzer *et al.* (1988) was associated with a *nit-3* rhythm. These authors used unbuffered media at approximately pH 5.6, which would have limited primary nitrogen uptake and thus *nit-3* would have become derepressed.

In contrast to its effect on photoconidiation, nutrient deprivation did not constrain injury-induced conidiation over time on PDA buffered to pH 2.8, rather it favoured it. Injury-induced conidiation was clearly observed on all plates, whereas photoconidiation was most intense on plates exposed to light at 48 h and decreased when light exposure

was later. Conidiation in the dark also increased over time in these experiments, which suggests that nutrient deprivation promotes injury-induced conidiation and conidiation in the dark and negatively regulates photoconidiation.

2.12.3 The Choice of Inoculum Differentially Affects Conidiation Phenotype

Photoconidiation over time at pH 2.8 was also investigated using mycelial plugs instead of conidial suspensions as inoculum. Conidiation was more advanced on the mycelial plug inoculum plates, which was attributed to the 12-24 h delay before conidial germination and growth, otherwise a similar pattern of conidiation in response to light was observed. In contrast, conidiation in the absence of stimuli did not intensify with age when mycelial inoculum was used. It is proposed that promotion of conidiation in the dark over time is related to nutrient deprivation. Together these results suggest that either nutrient deprivation does not occur on plates inoculated with mycelial plugs, which is highly unlikely, or deprivation does not promote conidiation and this is due to inoculum choice. There were two main points of difference between mycelial plugs and conidial suspensions as inoculum sources: age of the starting material and light exposure. Transfer of the plug to the test medium signalled the start of the experiment, however the hyphal cells on the plug and, therefore, at the centre of the resulting colony, were 2-3 d old. In contrast, there was a 12-16 h delay following inoculation before conidia germinated and hyphal cells began to appear. During preparation of the plates, hyphal cells on the mycelial plug were exposed to light, then returned to total darkness, whereas conidia germinated in the dark and, therefore, the resulting colonies were considered naïve to light. The experiment was repeated comparing plugs transferred under safe red light with those transferred under white light. Transfer of the plug under red light did not alter the phenotype, which suggests the source of the phenotypic difference may be due to the age of the inoculum. The choice of inoculum also affected conidiation in *T. harzianum* on PDA buffered from pH 2.8 to 3.2, where mycelial inoculum appeared to promote conidiation in the dark. In contrast to *T. harzianum*, the use of mycelial plugs appeared to inhibit dark conidiation in *T. atroviride*, suggesting that mycelial plugs differentially affect conidiation between these isolates. In *Fusarium oxysporum* the choice of inoculum has been shown to affect morphological variability (Awuah & Lorbeer, 1989). These authors demonstrated that variability increased significantly when mycelial plugs were used instead of conidial inoculum. In addition, as the cultures used for inoculation increased in age, so too did the resulting variability. Further work will be required to explore the exact cause of the phenotypic difference,

however these observations have obvious implications for experimental design and suggest that different inocula should be compared when evaluating *Trichoderma* spp. phenotypes.

2.12.4 Low pH Promotes Conidiation in *Trichoderma hamatum*

Photoconidiation was similarly affected by ambient pH in *T. hamatum* as observed in *T. atroviride*, however the optimal pH varied slightly and as observed in previous sections conidiation in response to a single light dose was severely constrained by an unknown variable. Unlike *T. atroviride*, photoconidiation remained constrained to the centre on the unbuffered PDA It was greatest on plates with an initial pH value from 2.8 to 3.2 and became sparser as the pH rose. Alkalisation of the medium also occurred on unbuffered PDA, however unlike *T. atroviride*, the pH was the same under the whole colony. On unbuffered PDA, photoconidiation in *T. atroviride* became constrained to the perimeter as the pH rose and this was partly attributed to the lower ambient pH at the hyphal front. It is possible that in the absence of an ambient pH gradient, conidiation occurs in the centre. The cause of the weak conidiation by *T. hamatum* was not determined, however this isolate has been demonstrated to conidiate in rings when grown under alternating light/dark conditions (Figure 2.1) and profusely on unbuffered standard PDA when grown in constant light (Appendix 7.3.1), which clearly demonstrates that under certain conditions this isolate conidiates well in response to light. Further work will be required to understand the variables constraining this isolate in comparison to other species of *Trichoderma*.

2.12.5 Colony Morphology is pH-Associated in *Trichoderma harzianum*

Growth and photoconidiation in response to increasingly acidic ambient pH was also investigated in *T. harzianum* and in accordance with our hypothesis, colony morphology was clearly pH-dependent on buffered media. On PDA buffered from pH 4.8 to 5.2 a spoke-wheel morphology was observed and this was absent on unbuffered PDA. In contrast to *T. atroviride* and *T. hamatum*, *T. harzianum* did not alkalise the media, indeed no significant changes in ambient pH were detected. This morphology was first observed in Section B on MM with glutamine or urea buffered to pH 5.4 and as observed in this section, it was absent on the unbuffered medium. Interestingly it was absent on MM with KNO_3. Together, these results suggest that in *T. harzianum* the spoke-wheel morphology is related to the buffering capacity, the ambient pH of the medium and possibly also to the presence of primary nitrogen.

Concurrent with the appearance of the spoke-wheel morphology, a yellow pigment was excreted into the medium, however unlike the spoke-wheel morphology it was also produced on the unbuffered medium and in a pH-dependent manner. This pigment has been observed before in Section A on unbuffered PDA at approximately pH 5.6 and in Section B on buffered and unbuffered MM with glutamine. Together, these results suggest production of this pigment is strictly pH-dependent and is not determined by the buffering capacity of the medium.

Most striking was the appearance of a stress-like morphology from pH 4.0 to 4.4 on buffered PDA. Growth was impaired and the colony margins were undulating resembling the stress-like morphology observed at higher pH values for *T. atroviride* on buffered MM with glutamine (2.7.6). The impairment of growth across a narrow range well within the limits for pH tolerance for this isolate was unexpected and to the best of our knowledge has not been described before. The stress morphology was also observed on unbuffered PDA, which suggests that like the yellow pigment, appearance of this morphology is strictly pH-dependent and not determined by the buffering capacity of the medium.

2.12.6 Low pH Promotes Conidiation in *Trichoderma harzianum*

Low pH promoted photoconidiation in *T. harzianum* in a similar fashion to *T. atroviride* and *T. hamatum*, which suggested photoconidiation may also be similarly regulated in the different species. On buffered PDA the optimal for photoconidiation was between pH 3.2 and 3.6 and no conidiation in response to light was observed above pH 4.0. A similar response was observed when mycelial plugs were used as inoculum, except photoconidiation was observed from pH 2.8 to 4.4, due to the increase in colony size. In contrast, photoconidiation was observed at all pH values on unbuffered media. On the basis of the photoconidiation experiments described in this section, we propose that low pH-dependent photoconidiation in isolates of *Trichoderma* is related to both the buffering state and the ambient pH of the medium.

On buffered media with conidial inoculum, a ring of conidia was formed subsequent to the photo-induced conidiation at the centre, which suggests that exposure to light affects cells produced subsequent to the light dose. The mechanism underlying this phenomenon is not known, however, light has been shown to trigger regulatory

pathways that have an effect on subsequent generations of cells that were not originally exposed to the light source. In *N. crassa*, for example, exposure to light stimulates circadian conidiation and pigment production (Linden *et al.*, 1997). This leads to secondary conidiation rings arising sequentially in roughly 24 h periods following the light event.

In *N. crassa*, circadian rhythm is controlled by the White Collar proteins WC-1 and WC-2 (Herrera-Estrella & Horwitz, 2007). The *T. atroviride* White Collar orthologues, BLR-1 and BLR-2 also regulate blue-light responses including photoconidiation, however no circadian association has been demonstrated for these transcription factors and in addition, no evidence of a light-induced circadian rhythm associated with photoconidiation has been found (Betina & Zajacová, 1978a; Deitzer *et al*., 1988; Schrüfer & Lysek, 1990; Section 1.4.2.3). It is tempting to speculate that the extra ring may be associated with a circadian rhythm and thus represents evidence for a light-induced circadian rhythm in *Trichoderma*.

2.12.7 Are the pH-related Responses PacC Regulated?

Transcriptional regulation of gene expression by the ambient pH is mediated in the filamentous fungi by the zinc finger protein transcription factor, PacC (Peñalva & Arst, 2002; 2004). Genes under the control of PacC are preferentially expressed either under acid or alkaline growth conditions, depending on the particular gene. As the ambient pH is lowered or raised the level of gene expression increases or decreases uniformly. Active alteration of the ambient pH by the fungus is considered a mechanism by which fungi exploit PacC regulation of various secreted enzymes, permeases and enzymes involved in the synthesis of exported metabolites such as toxins and antibiotics (Prusky & Yakoby, 2003; Peñalva & Arst, 2002). Gene expression can be regulated directly by PacC, indirectly by the effects of PacC regulation on other genes (Peñalva & Arst, 2002; 2004), or independently of PacC, such as through alterations in nutrient uptake rates (Szabo & Stofanikova, 2002).

Recent studies have linked PacC regulation to conidiation in the *T. harzianum* isolate, CECT 2413 (Moreno-Mateos *et al.*, 2007). Conidiation was 100-fold higher (10-fold on unbuffered) on mimimal medium agar at pH 5.5 than at pH 3.0 and growth rates were the same regardless of buffering. At pH 7.5, the growth rate was significantly reduced and conidiation was faint on both buffered and unbuffered plates. The *pacC* orthologue,

pac1 was isolated from *T. harzianum* and acid (loss of *pac1*) and alkaline (constititutive Pac1 activity) mimicry mutants created. Both mutants resembled the wild-type at pH 5.5 in growth and conidiation. At pH 3.3 the acid mimicking mutants resembled the wild-type, whereas at pH 7.5 conidiation was absent and growth was poor. In the alkaline mimicry mutants, conidiation resembled the wild-type at pH 7.5, but was absent at pH 3.3 and growth was also poor. The conidiation phenotypes of the acid and alkaline mimicking mutants strongly suggest Pac1 plays a regulatory role in conidiation.

The majority of experiments reported by Moreno-Mateos *et al.* (2007) were carried out using minimal medium with 2% glucose and 5 g/L ammonium sulphate (18.9 mM nitrogen), as the sole nitrogen source, whereas in this Section, the influence of the ambient pH was investigated on PDA. In Section B of this chapter, initial experiments on ambient pH were done on minimal medium with 2% glucose and 20 mM ammonium or amino acid based nitrogen, similar to that described in Moreno-Mateos *et al.* (2007). Buffering of the minimal medium negatively influenced conidiation in four of the five isolates tested, indeed in *T. atroviride* LU298, photoconidiation did not occur on buffered minimal medium at any pH (data not shown), whereas on buffered PDA, conidiation occurred in a disk from pH 2.8 to 3.6. Absence of conidiation on buffered minimal medium was the reason for choosing PDA as the test medium to investigate the influence of ambient pH on photoconidiation. On buffered PDA, no conidiation occurred above pH 3.6 in *T. hamatum*, above pH 4.0 in *T. atroviride* and above 4.4 in *T. harzianum*, whereas on unbuffered medium it occurred at all pH values tested (2.8 to 5.2). For this reason I suggest that the influence of ambient pH on buffered medium was not due to direct Pac1 regulation. Similarly, the spoke-wheel morphology observed in *T. harzianum*, only appeared on buffered medium, and was absent on the unbuffered medium, suggesting it is also independent of Pac1. Only the stress morphology and the production of the yellow pigment in *T. harzianum* LU675 were observed on both buffered and unbuffered medium in a pH-dependent fashion. It is possible that the stress morphology from pH 4.0 to 4.4 and excretion of the yellow pigment from pH 4.8 to 5.2 is Pac1 related, however growth in the relevant *Pac1* mutational backgrounds would be required to determine this.

SECTION D
PHOTOCONIDIATION ON BROTH-SOAKED FILTER PAPER

2.13 Introduction

All experiments presented in the study so far have used an agar medium, however considerable research has been done on photoconidiation using broth-soaked filter paper as the growth medium. In addition, molecular studies on genes involved in conidiation have used filter paper to evaluate photoconidiation phenotype. In response to single light exposure, both *T. viride* and *T. atroviride* have been reported to produce a single ring of profuse conidiation (Gressel and Galun, 1967; Casas-Flores *et al*., 2004). In this study, photoconidiation has occurred in both rings and disks and this was dependent on the isolate, the nitrogen status of the medium and/or the ambient pH and buffering capacity of the medium. Results from the previous section have led to the hypothesis that photoconidiation is promoted by high primary nitrogen and low pH and this determines the conidiation phenotype in response to light. The broth-soaked filter paper assays described in Chapter 1 predominantly used potato-dextrose broth (PDB) amended with yeast extract and casein hydrolysate (PDYC). Addition of yeast and casein would have increased the nutrient content of the medium, however only 2-3 mL of broth were added to the filters compared with 25-30 mL agar. Experiments using broth-soaked filter paper are, therefore, at a lower nutrient status compared with agar assays. In the previous sections, it was postulated that nutrient deprivation promotes photoconidiation in a ring even at low pH values, which suggests that the production of a photoconidiation disk on filter paper is unlikely. In this section, all five *Trichoderma* isolates under study were subjected to broth-soaked filter paper photoconidiation assays. On the basis of the literature and these studies, it was hypothesised that all isolates would produce a ring of conidia in response to a single light dose, except for *T. hamatum*, which would be unlikely to photoconidiate, due to its weak conidiation ability.

In Section C, an intimate relationship between the ambient pH and morphogenesis was demonstrated in three isolates of *Trichoderma.* Ambient pH effects on photoconidiation were postulated to be due to intracellular acidification and the influence of the ambient pH on nutrient uptake and availability. In this section the influence of ambient pH on photoconidiation was investigated in *T. atroviride* and *T. harzianum* using broth-soaked filter paper. It was hypothesised that photoconidiation would be similarly regulated on a

filter paper medium, but photoconidiation would occur in a ring due to the lower nutrient status.

2.14 Materials and Methods

2.14.1 Blue-light Photo-induction of Conidiation on Broth-soaked Filter Paper

Trichoderma hamatum LU592, *T. atroviride* LU298, *T. asperellum* LU697, *T. virens* LU540 and *T. harzianum* LU675 were subjected to a filter paper photoconidiation assay essentially as described in Berrocal-Tito *et al.* (1999). Inoculum plugs from 2 d old *Trichoderma* spp. cultures grown on PDA with 0.2% yeast extract and 0.12% casein hydrolysate (PDYC agar) or PDA were inoculated centrally to filter papers (70 mm Whatman #1 overlaid with an 80 mm Whatman #50), which had been soaked in PDB with 0.2% yeast extract and 0.12% casein hydrolysate (PDYC) or PDB, respectively, and placed in 9 mm Petri dishes. Plates were incubated unsealed at 23°C in total darkness for 36 h, exposed to blue light as described in section 2.2.3 and then incubated for a further 72 h in total darkness. Control plates were incubated in total darkness for the duration of the experiment. Four plates per treatment were inoculated and both experiments were repeated once.

In a third experiment photoconidiation was investigated in *T. harzianum* LU675 on PDYC-soaked filter paper, as described above, except comparison was made between plates prepared under standard lighting versus plates prepared under safe red light.

2.14.2 Photoconidiation in *T. atroviride* and *T. harzianum* at pH 2.8 to 5.2 on PDYC-soaked Filter Paper

The effect of pH on photoconidiation was investigated in *T. atroviride* LU298 and *T. harzianum* LU675 using a modification of the filter paper photoconidiation assay. Filter papers were soaked in either pH-buffered or unbuffered and pH adjusted PDYC broth, which ranged in value from pH 2.8 to 5.2 with intervals of 0.4. In previous experiments on pH-buffered agar the growth rates of both isolates were reduced and this was most pronounced in *T. harzianum* (sections B and C). Therefore, to ensure all colonies were of reasonable size and for consistency, the light dose was given at 42 h instead of 36 h on both the buffered and unbuffered filter papers, and control plates were incubated for 4.75 d (114 h) instead of 4.5 d (108 h). Buffered PDYC broth was prepared as follows: PDB at 2 X concentration with 0.4% yeast extract and 0.24% casein hydrolysate were

dissolved in the appropriate McIlvaine buffer (citrate/phosphate) (Appendix 7.2.3), then the pH was adjusted with HCl and an equivalent volume of water was added prior to autoclaving. Unbuffered PDYC broth was prepared in a similar fashion, except the PDB, yeast extract and casein hydrolysate were dissolved in water rather than citrate/phosphate buffer. PDYC broth-soaked filter papers were inoculated as described in 2.14.1 and incubated unsealed at 23°C in total darkness for 42 h, then exposed to blue light for 15 min as described in 2.2.3 and incubated for a further 72 h in total darkness. Control plates were incubated in total darkness for the duration of the experiment. Two plates per treatment were inoculated.

2.14.3 Photoconidiation in *T. atroviride* and *T. harzianum* at pH 2.8 to 5.2 on PDB-soaked Filter Paper

The effect of pH on photoconidiation in *T. atroviride* LU298 and *T. harzianum* LU675 was also investigated using pH-buffered and unbuffered PDB, which ranged in value from pH 2.8 to 5.2 with intervals of 0.4. As described in 2.14.2, light was given at 42 h for consistency and control plates incubated a further 6 h. Buffered and unbuffered PDB was prepared as described for PDYC broth in 2.14.3, except the yeast extract and casein hydrolysate were omitted. PDB-soaked filters were inoculated as described in 2.14.1 and incubated unsealed at 23°C in total darkness for 42 h, then exposed to blue light for 15 min as described in 2.2.3 and incubated for a further 72 h in total darkness. Control plates were incubated in total darkness for the duration of the experiment. Two plates per treatment were inoculated.

2.14.4 Analysis of Rhythmic Conidial Production in *T. harzianum*

The phenomenon of circadian rhythm-associated conidiation was investigated in *T. harzianum* isolate LU675 using a modification of the filter paper photoconidiation assay. Mycelial plugs from 2 day old cultures were inoculated to three 120 mm Whatman #1 filters, which had been soaked in PDYC and placed in 150 mm Petri dishes. Plates were incubated unsealed at 23°C in total darkness for 48 h, then exposed to blue light for 15 min, as described in 2.2.3 and then incubated for a further 96 h in total darkness. Control plates were incubated in total darkness for the duration of the experiment. Four plates per treatment were inoculated and the experiment was repeated once.

2.15 Results

2.15.1 Blue-light Photo-induction of Conidiation on Broth-soaked Filter Paper

2.15.1.1 Photoconidiation on PDYC

Five *Trichoderma* isolates were subjected to a standard filter paper photoconidiation assay on PDYC-soaked filter paper. The control plates were incubated in total darkness for the duration of the experiment and thus represented 4.75 days (114 h) growth. No conidiation was observed in *T. hamatum* on the PDYC control plates (Figure 2.25A). Conidiation was observed on the *T. atroviride* control plates and this was sparse and localised on and around the mycelial plug. Conidia were colourless to green in pigmentation. In addition, a distinctive coconut odour was detected indicating 6-PAP production (Sivasithamparam & Ghisalberti, 1998). Green pigmented conidia were also observed on and around the mycelial plug on the *T. asperellum* control plates, though more intense than that observed in *T. atroviride.* On the *T. virens* control plates, conidia were produced on the mycelial plug, however, unlike *T. atroviride* and *T. hamatum*, a diffuse ring of conidia was produced at a diameter of about 25 mm. Similarly, on the *T. harzianum* control plates, conidia were observed on the mycelial plug and in a ring at about 20 mm in diameter. Conidia were dark green in pigment. In addition, a yellow pigment was secreted onto the filter paper and this pigment appeared to be absent inside the conidial ring. The experiment was repeated using *T. harzianum* only and comparison made between plates prepared in the light and plates prepared under safe red lighting. Conidiation occurred in a ring at the centre of the control plate regardless of preparation conditions, which demonstrated the ring was not light-induced.

In response to a single blue-light exposure, all isolates, except *T. virens*, produced a ring of conidia, which correlated with the position of the colony margin at the time of light exposure. In contrast, on the PDA photoconidiation treatment plates (2.3.2) only three of the five isolates produced a clearly observable conidiation ring, whereas *T. hamatum* sparsely conidiated at the centre of the plate only and *T. harzianum* produced a disk of conidia. On the *T. hamatum* PDYC-filter treatment plates a 3 mm thick ring of mainly unpigmented conidia was produced in response to light (Figure 2.25B). Conidiation was not dense and in some parts the ring was discontinuous. In contrast to the control plates, conidiation was observed on the mycelial plug. A 3 mm wide ring of conidiation was also produced on the *T. atroviride* treatment plates, though conidiation was more mature and dense compared with *T. hamatum*. In addition, random sparse conidiation was observed within the ring to 5 mm before the mycelial plug, which gave the appearance

of a faint second ring. Conidiation on the mycelial plug was greater than on the control plates. In addition, the coconut odour was more intense compared with the control plates. In *T. asperellum*, a slightly thicker (4 mm), though more diffuse, ring of green conidia was produced in response to light. Conidiation occurred randomly inside the ring, though unlike *T. atroviride*, conidiation occurred up to the position of the mycelial plug. Conidiation on the plug was similar to the control plates. The *T. virens* treatment plates were indistinguishable from the controls. In *T. harzianum*, a diffuse outer ring of green conidia was produced, which correlated with the position of the colony margin at the time of light exposure and, in addition, there was a central ring similar to that observed on the control plates. A small amount of conidiation was also observed at the edge of the filter paper. A yellow pigment was secreted onto the filter paper, as previously described on the control plates, and the presence of the pigment appeared to alternate with the positions of the conidial rings (Figure 2.26).

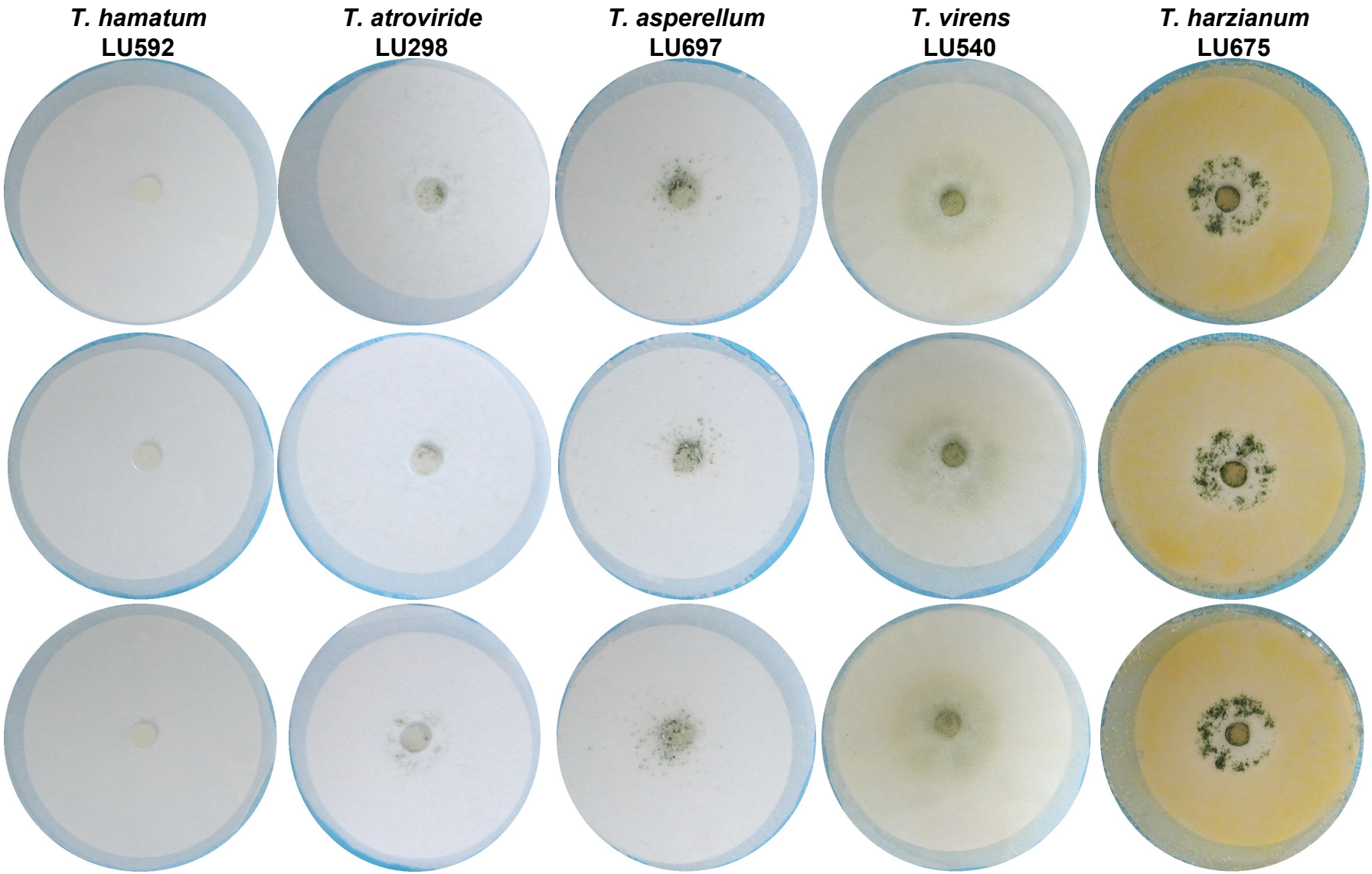


Figure 2.25A. Photoconidiation experiment (control plates) on PDYC-soaked filter. Cultures were grown for 4.5 d in total darkness. Three replicates from the first experiment are presented.

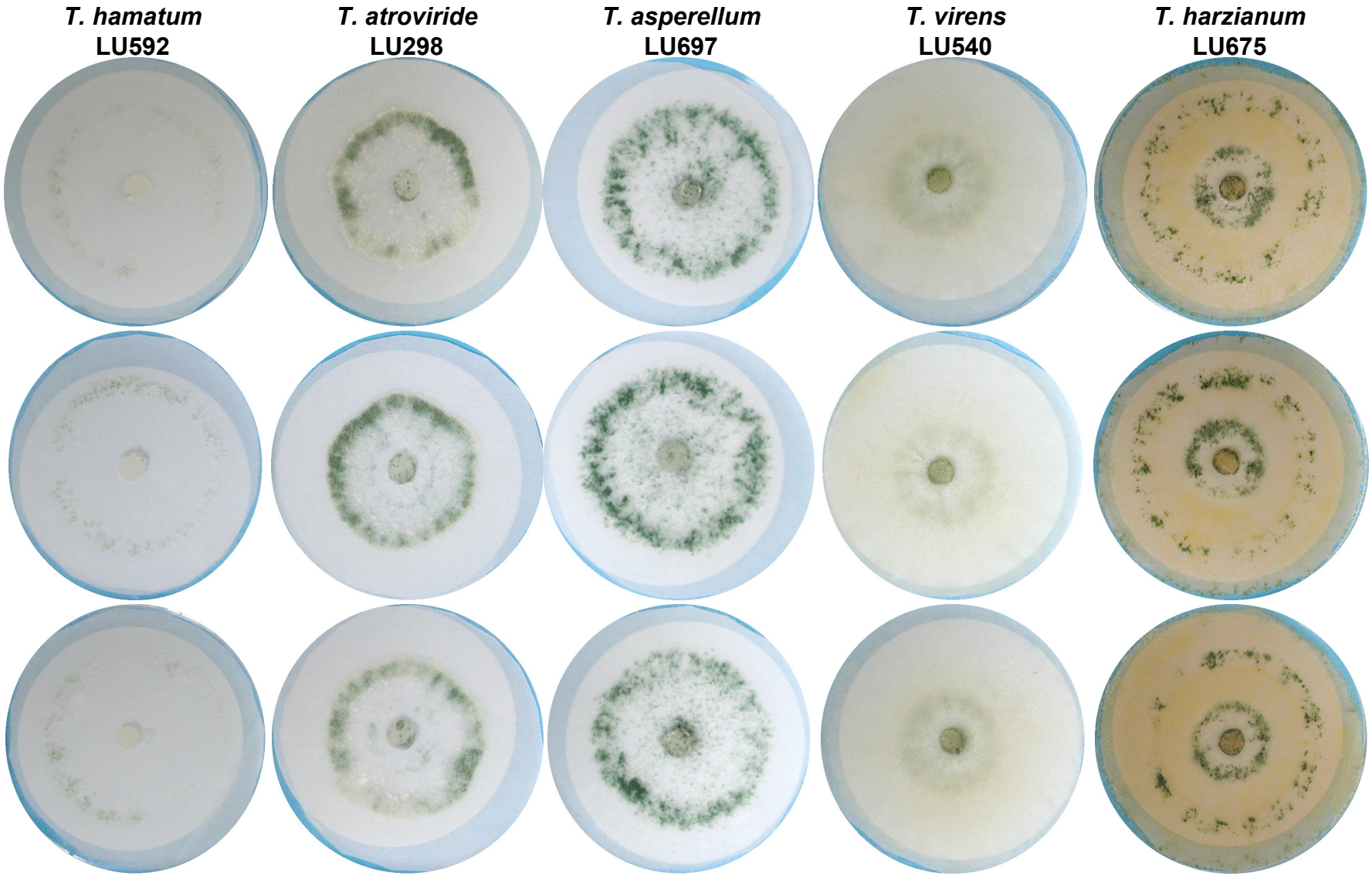


Figure 2.25B. Photoconidiation experiment (treatment plates) on PDYC-soaked filter. Cultures were grown for 36 h in total darkness, then photoinduced and grown a further 72 h in total darkness. Three replicates from the first experiment are presented.

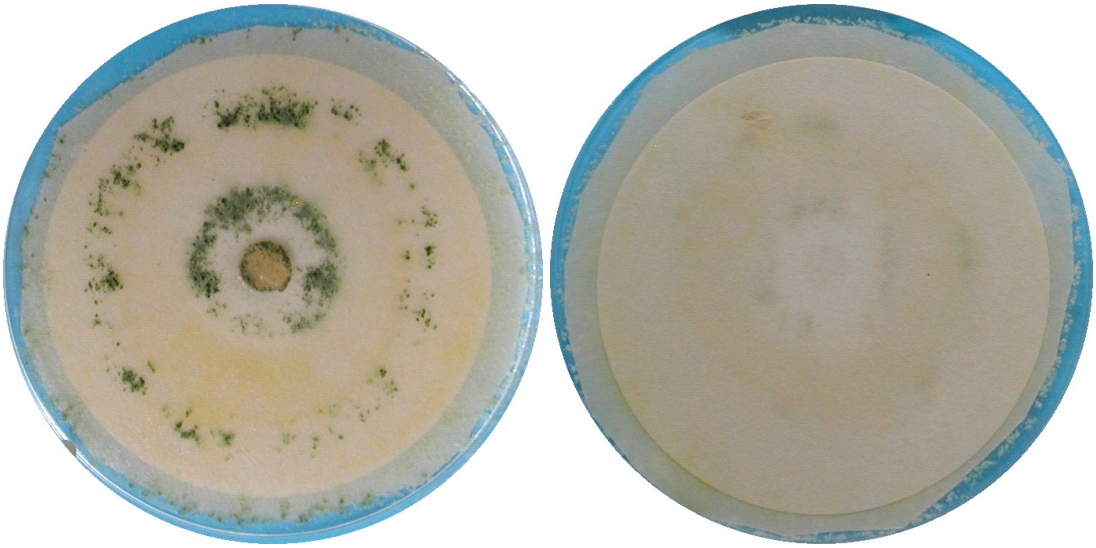


Figure 2.26. Enlargement of *T. harzianum* PDYC treatment plate showing banding of pigment distribution. A. Top view of plate. B. Reverse view of plate

2.15.1.2 Photoconidiation on PDB

In addition to the standard PDYC broth, photoconidiation was also assessed on filter paper soaked in PDB. No conidiation was observed on the *T. hamatum* 4.5 d old PDB control plates (Figure 2.27A), similar to that described for PDYC. Conidiation on and around the mycelial plug was more intense on the *T. atroviride* PDB control plates than observed in the PDYC experiment. Conidia on the plug were green in pigment. Around the plug, conidia were colourless to green and were arranged in a ring-like fashion. Similar to the PDYC experiment, a coconut odour was detected. Conidiation was also observed on the *T. asperellum* control plates and this was more mature in pigmentation than that observed on the PDYC controls. Similar to the PDYC experiment, conidiation was observed on the mycelial plug of the *T. virens* control plates, however the diffuse ring of conidia observed on PDYC was barely visible on PDB. A yellow pigment was observed on the filter paper and there appeared to be a small zone around the plug where no pigment was produced. This is the first observation of this pigment in *T. virens* LU540. Conidia were formed on the mycelial plugs from the *T. harzianum* PDB control plates and similar to *T. virens* this differed from the PDYC experiment in that conidiation around the mycelial plug was barely visible. A yellow pigment was observed on the filter and as described on the PDYC controls, there appeared to be a pigment free zone at the centre of plate. Pigment production was noticeably more intense on the PDB controls compared with PDYC.

All cultures conidiated in response to light when grown on PDB. Conidiation correlated with the position of the colony margin at the time of light exposure in all cultures except *T. virens* and *T. harzianum*. In the PDYC experiment, conidiation correlated with the colony margin at the time of light exposure in all cultures except *T. harzianum.* In *T. hamatum*, a ring of green conidia was formed on the PDB treatment plates, which were more mature than observed on the *T. hamatum* PDYC treatment plates (Figure 2.27B). In *T. atroviride*, the growth rate was faster on PDB compared with PDYC and therefore the conidial ring was noticeably larger in diameter. Similar to that observed in *T. hamatum*, conidial pigmentation was more mature compared with the *T. atroviride* PDYC treatment plates. The coconut odour was more intense compared with the controls, which was also observed in the PDYC experiment. The conidial ring on the *T. asperellum* treatment plates was narrower and less dense compared with the PDYC treatment plates. A sparse ring of conidia was produced in response to light on the *T. virens* plates similar to the PDYC treatment plates and the diameter of the ring was smaller than the colony margin at the time of light exposure. Faint pigment production outside the central zone was also observed. A ring of green conidia was produced on the *T. harzianum* treatment plates in response to light. This was the same as the inner ring of conidia observed on the PDYC treatment plates, which did not correlate with the colony margin at the time of light exposure. Similar to the PDYC treatment plates, sparse conidiation was observed at the edge of the filter paper and a yellow pigment was observed on the filter paper.

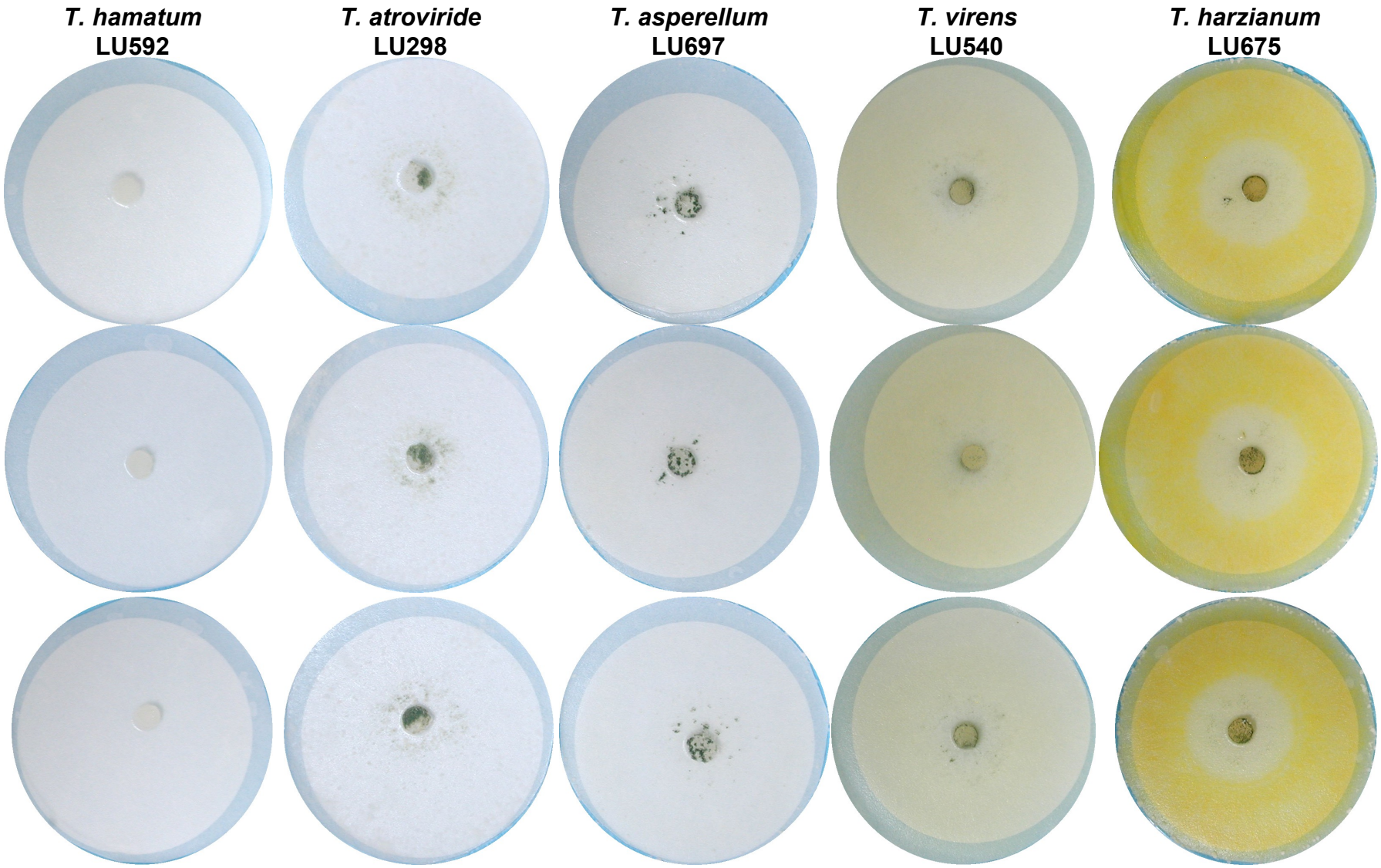


Figure 2.27A. Photoconidiation experiment (control plates) on PDB-soaked filter paper. Cultures were grown for 4.5 d in total darkness. Three replicates from the first experiment are presented.

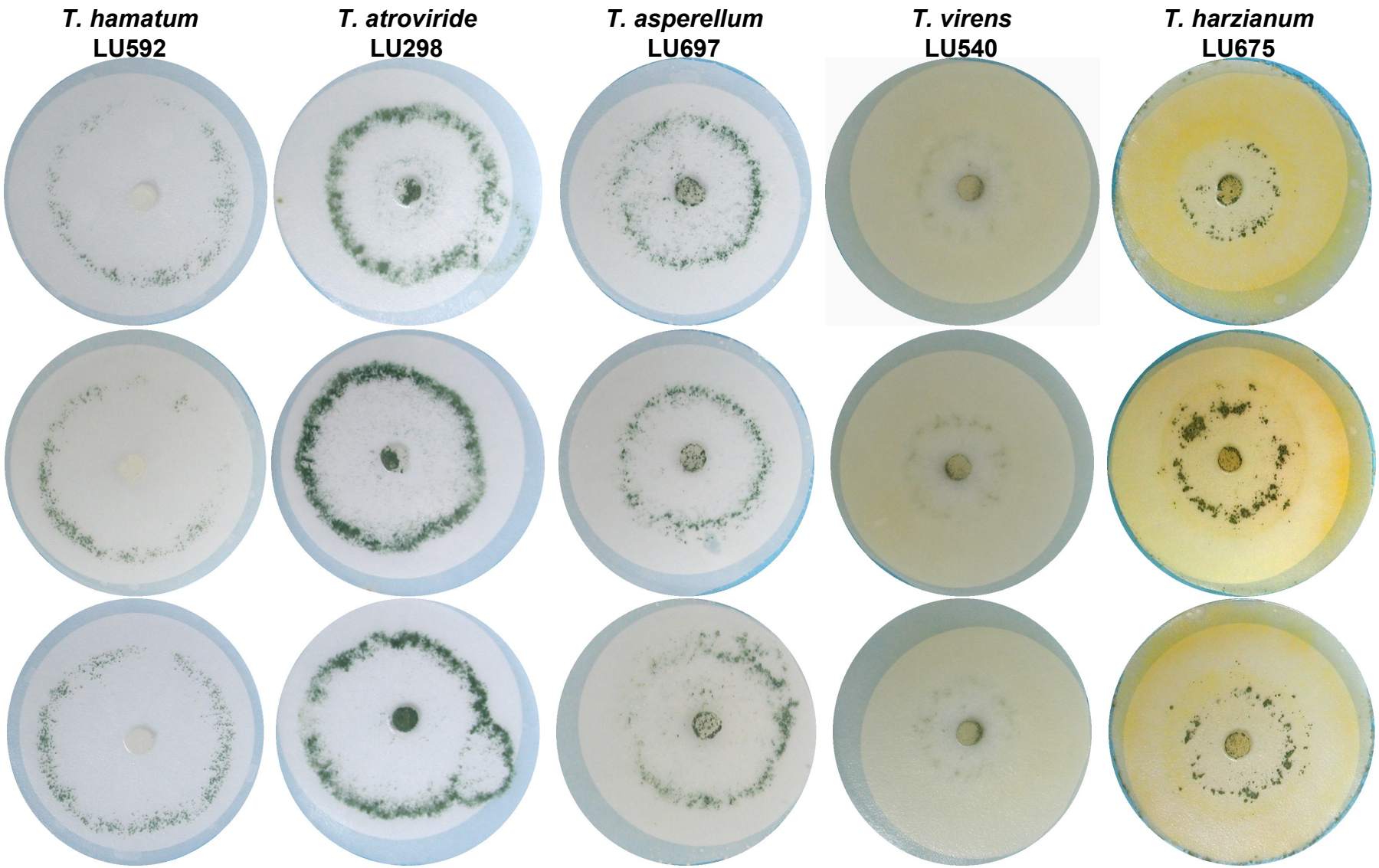


Figure 2.27B. Photoconidiation experiment (treatment plates) on PDB-soaked filter paper. Cultures were grown for 36 h in total darkness, then photoinduced and grown a further 72 h in total darkness. Three replicates from the first experiment are presented.

2.15.2 Conidiation Induction on PDYC Filter Paper from pH 2.8 to 5.2

2.15.2.1 *Trichoderma atroviride*

pH-buffered PDYC Broth

In contrast to the conidiation ring observed in the first experiment with PDYC broth (2.11.1.1), no photoinduced conidiation was observed in *T. atroviride* at any value on the pH-buffered PDYC broth-soaked filter paper (Figure 2.28A and 2.28B). Sparse conidiation was barely discernible on the mycelial plugs at pH 2.8 and 3.2, otherwise there was no discernible difference between controls and treatments and between pH values. These results also differed from the pH-buffered PDA experiment (2.11.1.1), where background and profuse light-induced conidiation was observed in *T. atroviride* in a low pH-dependent manner. No variation was observed between the replicates in all PDYC pH-filter assays.

Unbuffered PDYC Broth

No conidiation was observed on the control filter papers, however sparse conidiation was observed on the mycelial plugs (Figure 2.29A and 2.29B). Similar to the first PDYC (2.11.1.1) experiment and in contrast to the pH-buffered light treatment plates above, a ring of conidia which correlated with the position of the colony margin at the time of light exposure was produced in response to light on the pH 4.0 to 5.2 treatment plates. Pigmentation ranged from colourless to green and the most profuse and mature conidiation was observed at pH 4.4. This differed from the unbuffered, pH adjusted PDA experiment (2.11.2.1) in which conidiation occurred at all pH values, was disk-like and most profuse and mature at pH 3.6.

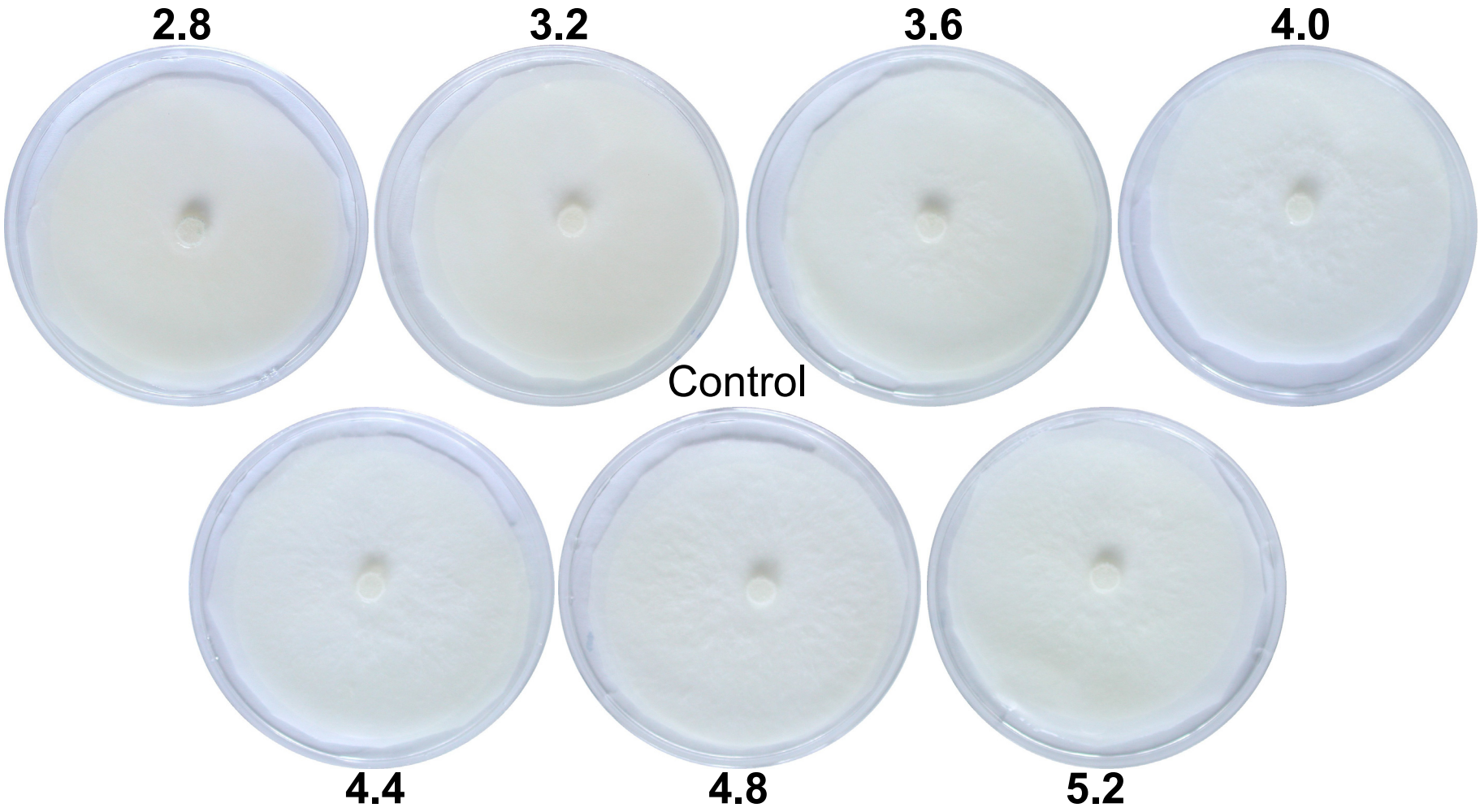


Figure 2.28A. *Trichoderma atroviride* photoconidiation experiment (control plates) on pH buffered (2.8 to 5.2) PDYC broth-soaked filter paper. Cultures were grown for 4.75 d (114 h) in total darkness.

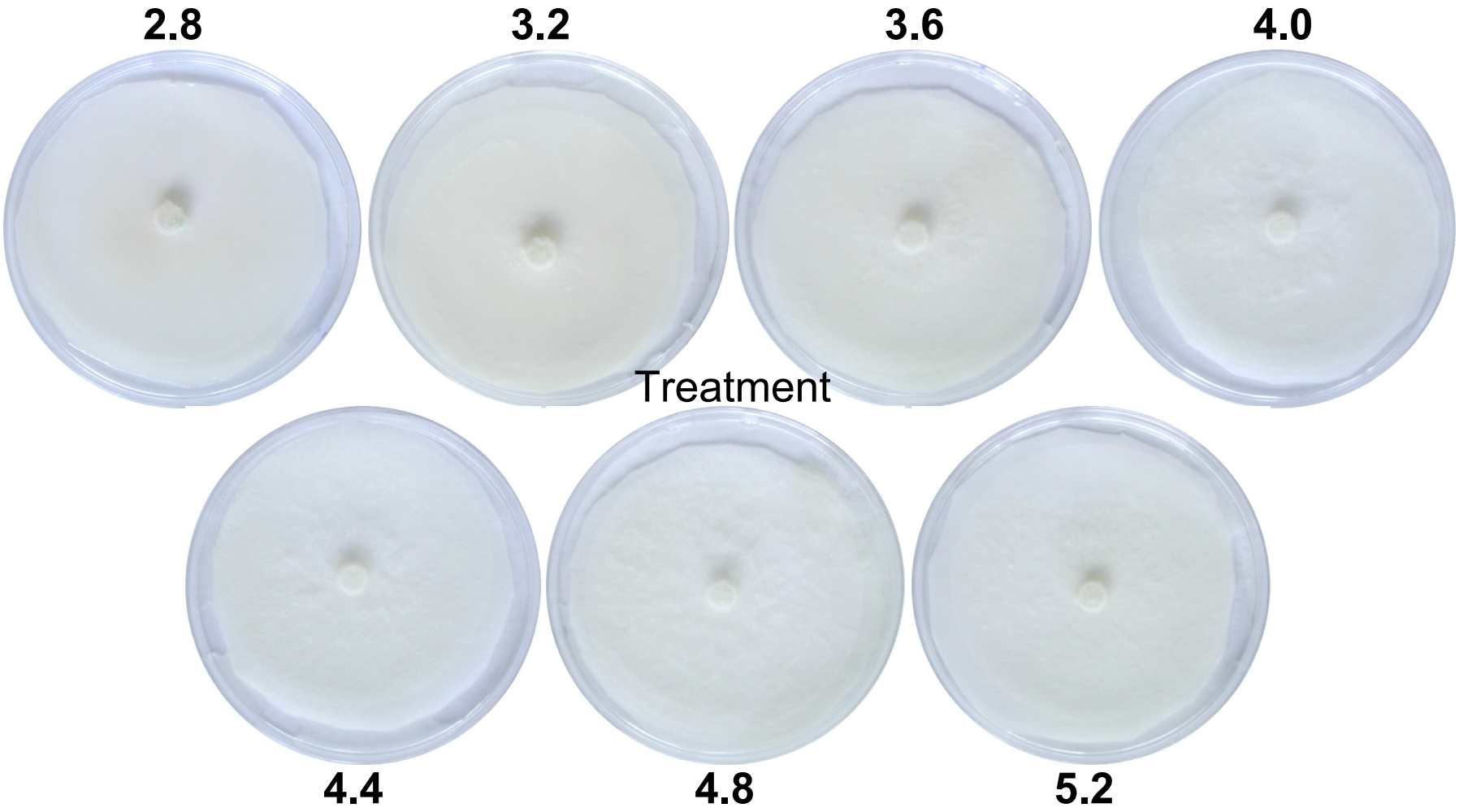


Figure 2.28B. *Trichoderma atroviride* photoconidiation experiment (treatment plates) on pH buffered (2.8 to 5.2) PDYC broth-soaked filter paper. Cultures were grown for 42 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

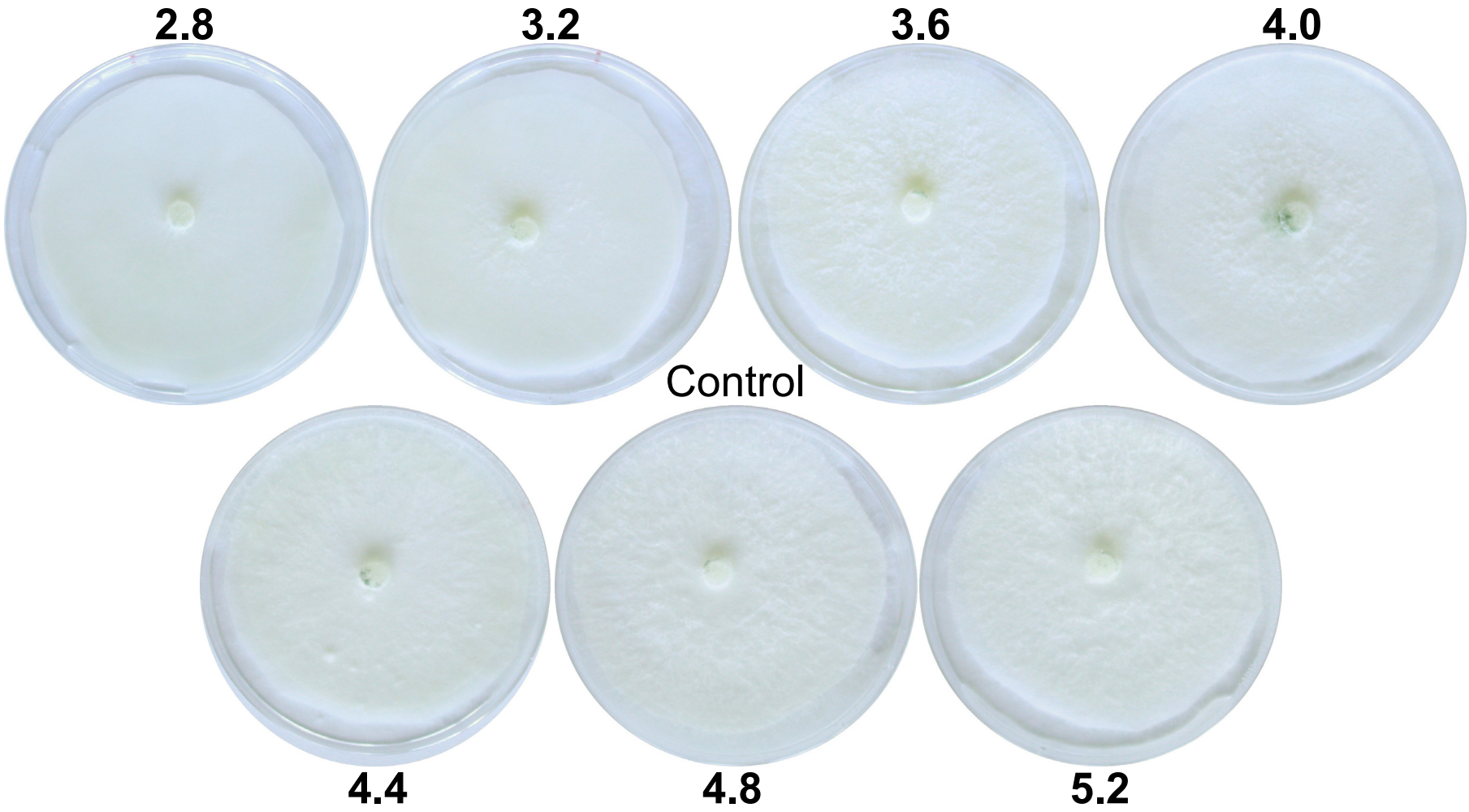


Figure 2.29A. *Trichoderma atroviride* photoconidiation experiment (control plates) on unbuffered, pH adjusted (2.8 to 5.2) PDYC broth-soaked filter paper. Cultures were grown for 4.75 d (114 h) in total darkness.

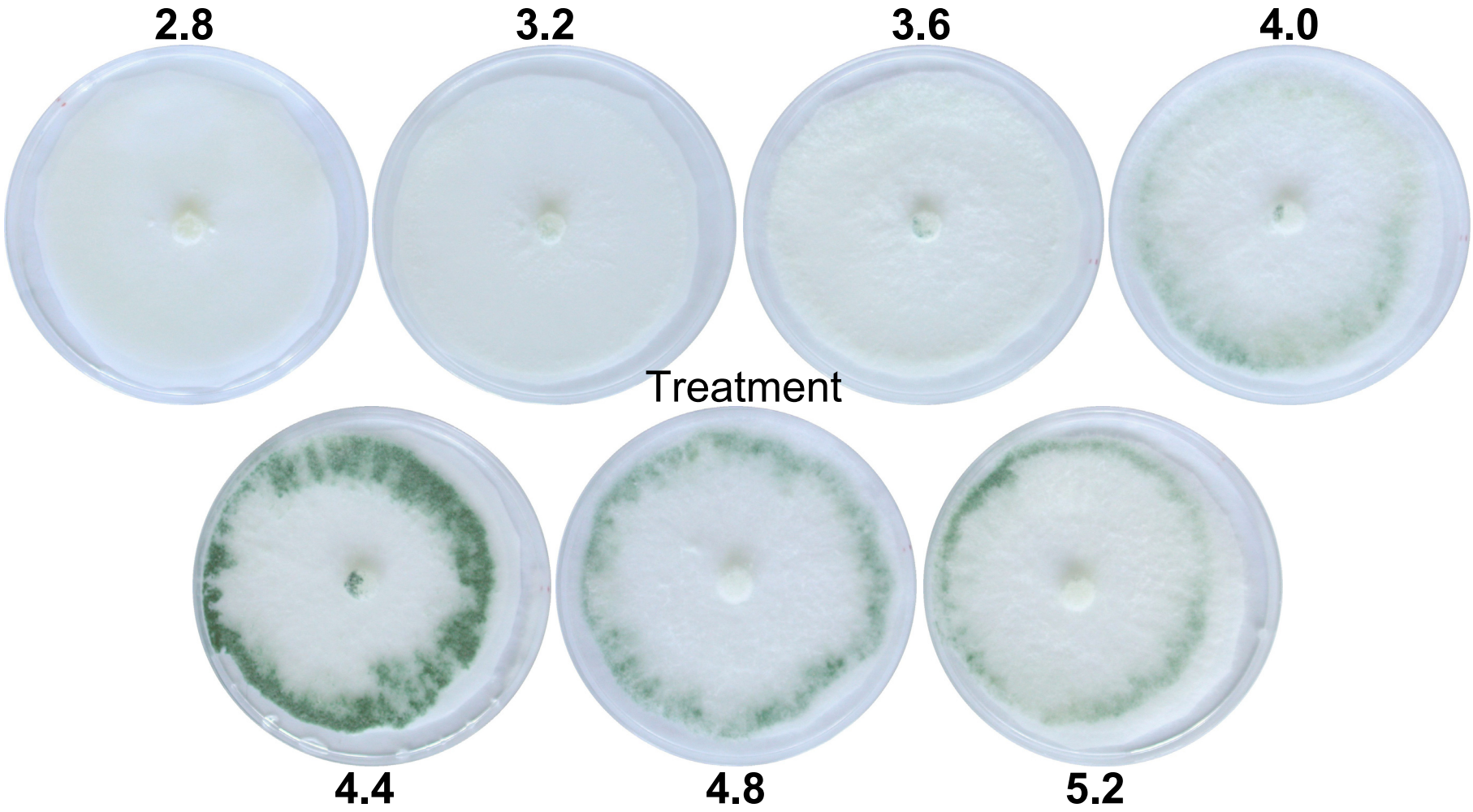


Figure 2.29B. *Trichoderma atroviride* photoconidiation experiment (treatment plates) on unbuffered, pH adjusted (2.8 to 5.2) PDYC broth-soaked filter paper. Cultures were grown for 42 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

2.15.2.2 *Trichoderma harzianum*

pH-buffered PDYC Broth

In contrast to *T. atroviride*, both background conidiation and photoconidiation were observed in *T. harzianum* grown on pH-buffered PDYC-soaked filter paper. Similar to that observed on the pH-buffered PDA experiments, distinct colony morphologies were observed and the most striking was the pH-dependent production of the yellow pigment. At pH 2.8 green conidia were observed on the mycelial plug and sparse colourless conidiation was observed on the filter paper near the plug (Figure 2.30A). At pH 3.2 and 3.6, conidiation was slightly more intense and dispersed randomly across the plate. On the pH 4.0 control plates, conidiation was green in pigment and restricted to the immediate vicinity of the plug. In addition, the colony margin was clearly uneven. The uneven appearance of the margin was also observed on the pH 4.4 and 4.8 plates and this was most pronounced at pH 4.4. Conidiation was observed around the mycelial plug on the pH 4.8 control plates and this was arranged in a ring-like fashion. Similar to that observed in the first experiment with PDYC broth (2.11.1.1), a yellow pigment was secreted onto the filter paper at pH 4.8 and pH 5.2. A yellow pigment was also observed from pH 4.8 to 5.2 in the pH-buffered PDA photoconidiation experiments (2.11.5). Immature conidia were barely discernible on the mycelial plug at pH 5.2.

In response to light, *T. harzianum* conidiated on pH-buffered PDYC in a pH-dependent fashion. In a trend similar to that observed on pH-buffered PDA using both conidial suspension and mycelial plug inoculum (2.11.5), photoconidiation was most intense at the lower pH values decreasing significantly as the pH rose and from pH 4.8 to 5.2 a yellow pigment was produced onto the filter paper. At pH 2.8 a disk-like cluster of green conidia were produced that correlated with the position of the colony margin at the time of light exposure (Figure 2.30B). At pH 3.2, a ring of green conidia was produced that also correlated with the colony margin at light exposure. At pH 3.6, sparse conidiation was observed though not readily discernible as a ring and the outer limits of the conidiation zone also correlated with the margin at light exposure. A similar pattern of conidiation was observed at pH 4.0 though conidiation was considerably reduced. From pH 4.4 to 5.2, there was no discernible difference between the control and treatment plates.

Unbuffered PDYC Broth

Mature green conidia were produced on all control plates and though more intense than on the buffered controls a similar pattern of conidiation was observed. On the pH 2.8 control plates, a diffuse cluster of predominantly green conidia was observed on and near to the mycelial plug (Figure 2.31A). At pH 3.2 and 3.6, conidiation was significantly more profuse and green conidia were observed across the plate. At pH 4.0, conidiation was restricted to and near the mycelial plug. Conidiation was also observed at the vicinity of the plug on the pH 4.4 control plates and, in addition, unpigmented and pigmented conidia were observed at the edge of the filter paper. A yellow pigment was observed at pH 4.4 in a narrow ring surrounding the central cluster of conidia and also at the filter edge. At pH 4.8, conidia were again clustered at the centre and colourless to green conidia were observed at the edge of the filter paper. The yellow pigment was present across the plate. Green conidia were observed at the centre of the pH 5.2 plates and colourless to green conidia at the filter paper edge. The yellow pigment was produced in abundance except within the conidiation zone in the centre where it was absent. A similar pattern of conidiation and pigment production was observed on the *T. harzianum* control plates in the first PDYC experiment (2.15.1.1).

Additional mature conidiation was produced in response to light on unbuffered PDYC broth-soaked filter paper. At the lowest pH values, background conidiation gave the ring a disk-like appearance but as the pH rose, conidiation became less diffuse and more concentrated into a ring. Conidiation correlated with the colony margin at the time of light exposure. A similar pattern of conidiation was observed in the unbuffered, pH-adjusted PDA experiment (2.11.4.3). From pH 2.8 to 3.6, a diffuse ring of green conidia was observed in addition to the background conidiation (Figure 2.31B). Two rings in addition to the ring-like background conidiation in the centre were observed on the pH 4.0 treatment plates. The outer ring was thicker then the inner ring and correlated with the margin at light exposure. In addition the outer ring displayed a ridge like pattern similar to the *T. harzianum* ring produced in the first PDYC experiment (Figure 2.29B). A similar pattern of conidiation was observed on the pH 4.4 plates, though the outer ring was more defined and intense compared with pH 4.0 and the inner ring was very sparse. In addition, the ring of yellow pigment observed on the pH 4.4 control plates was also present on the treatments. At pH 4.8 conidiation was slightly more intense and defined compared with pH 4.4 and the yellow pigment observed on the controls was present. Similar to the *T. harzianum* treatment plates in the first experiment, the pigment

appeared to alternate with the two conidial rings. Pigment production and conidiation also appeared to alternate on the pH 5.2 treatment plates, however the outer ring was less intense and the ring was discontinuous and extended for half of the plate only.

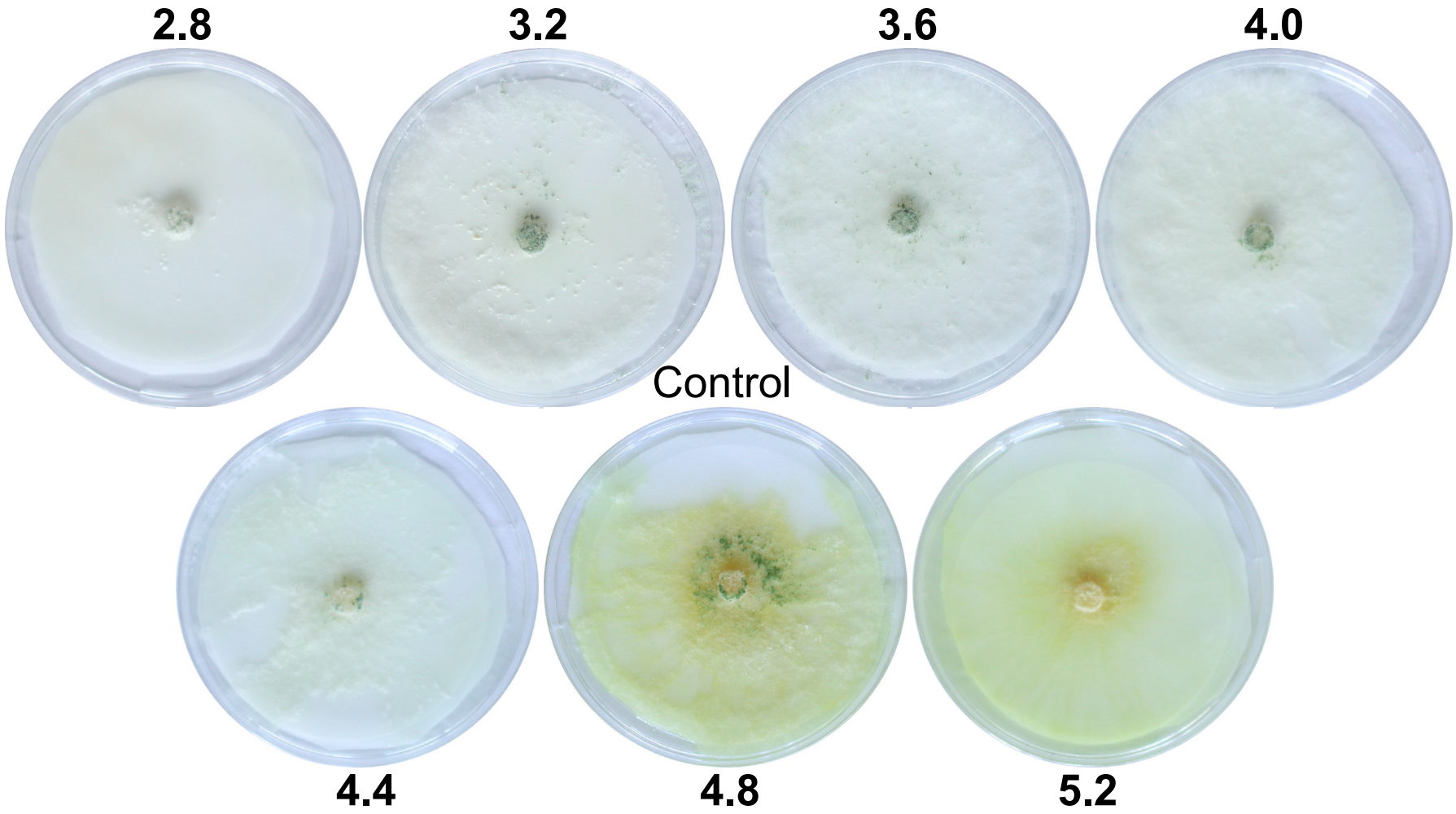


Figure 2.30A. *Trichoderma harzianum* photoconidiation experiment (control plates) on pH-buffered (2.8 to 5.2) PDYC-soaked filter paper. Cultures were grown for 4.75 d (114 h) in total darkness.

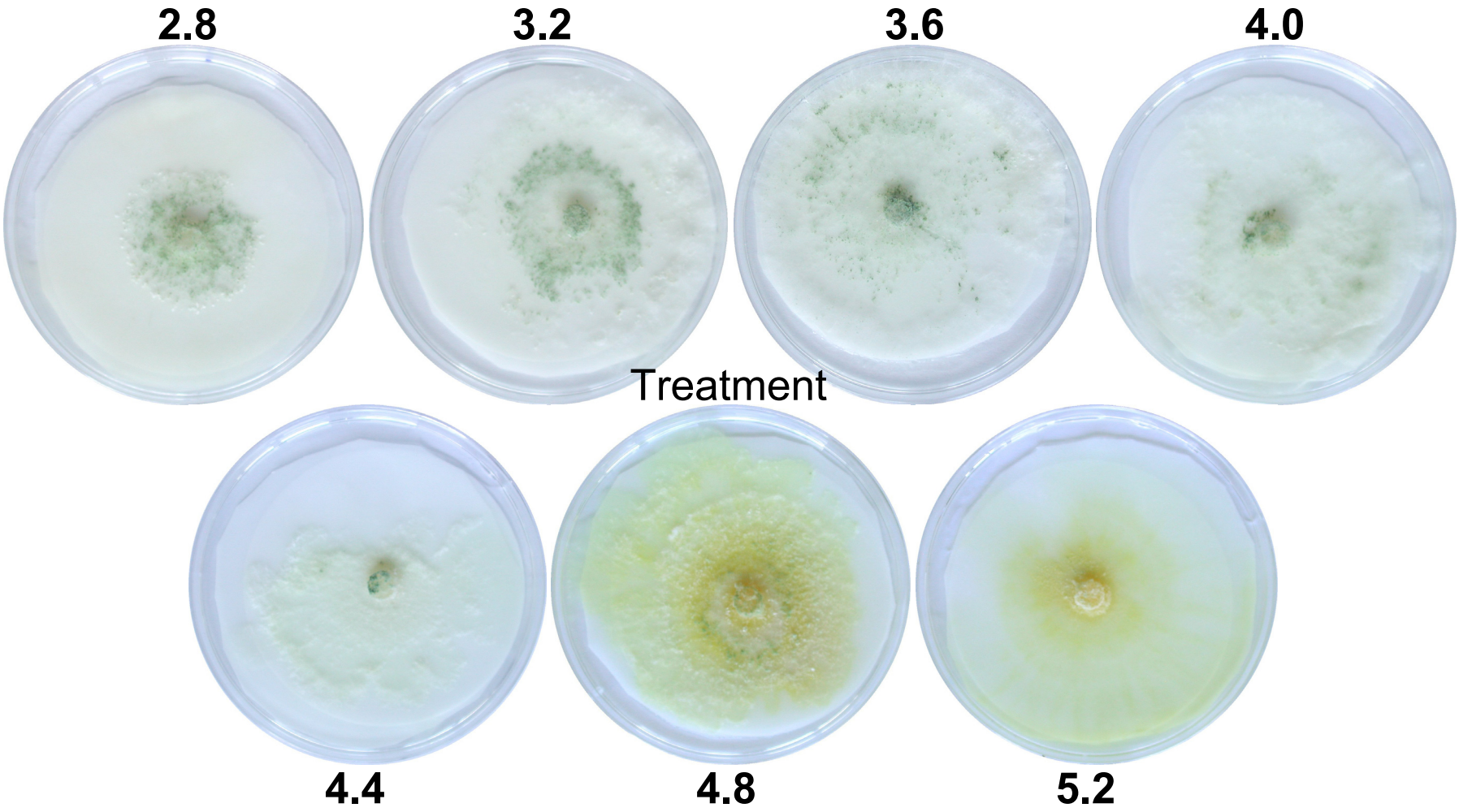


Figure 2.30B. *Trichoderma harzianum* photoconidiation experiment (treatment plates) on pH-buffered (2.8 to 5.2) PDYC-soaked filter paper. Cultures were grown for 42 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

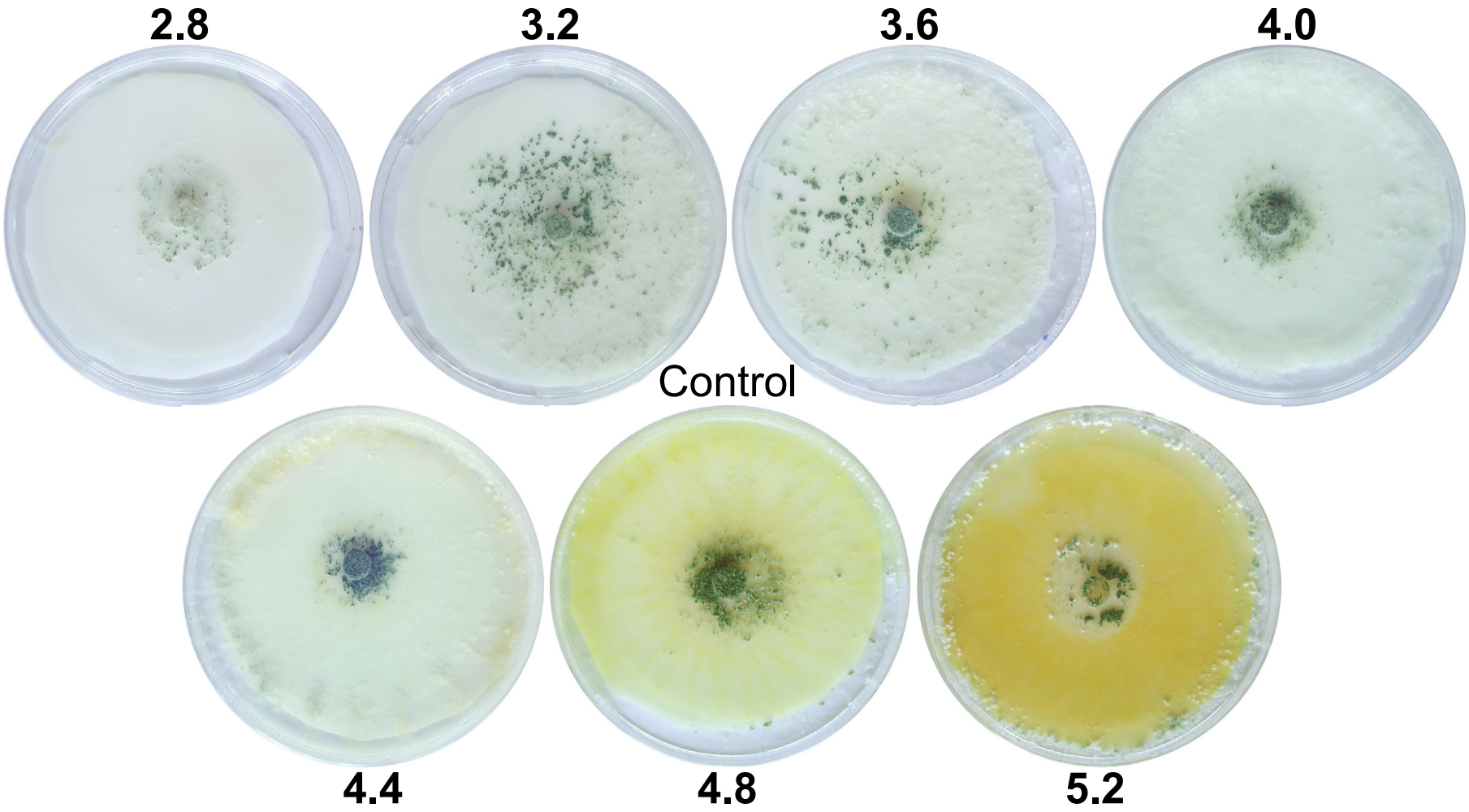


Figure 2.31A. *Trichoderma harzianum* photoconidiation experiment (control plates) on unbuffered, pH adjusted (2.8 to 5.2) PDYC broth-soaked filter paper. Cultures were grown for 4.75 d (114 h) in total darkness.

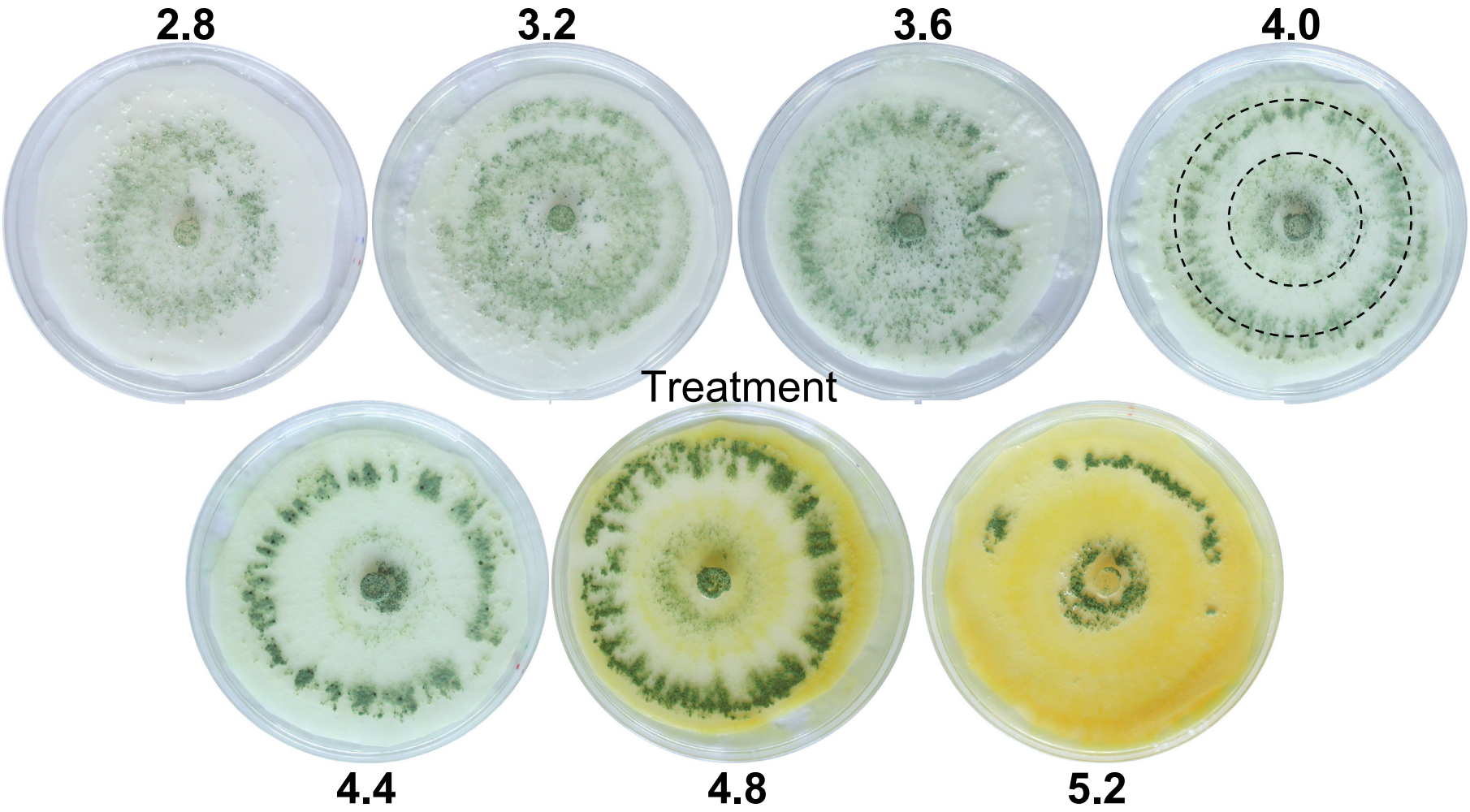


Figure 2.31B. *Trichoderma harzianum* photoconidiation experiment (treatment plates) on unbuffered, pH adjusted (2.8 to 5.2) PDYC broth-soaked filter paper. Cultures were grown for 42 h in total darkness, then photoinduced and grown a further 72 h in total darkness. the two rings at pH 4.0 are indicated by dashed circles.

2.15.3 Conidiation Induction on PDB Filter Paper from pH 2.8 to 5.2

In this experiment the effect of pH on photoconidiation in *T. atroviride* and *T. harzianum* was investigated on filter paper soaked in buffered and unbuffered PDB ranging from pH 2.8 to 5.2. The experimental conditions were the same as for the PDYC pH experiment and no variation was observed between the replicates.

2.15.3.1 *Trichoderma atroviride*

pH-buffered PDB

In contrast to the first PDB photoconidiation experiment (2.15.1.2) and to the pH-buffered PDA plate assays (2.11.1.2) and similar to the pH-buffered PDYC experiment (2.15.1.1), no conidiation in response to light was observed in *T. atroviride* at any pH, that is, the treatment plates were indistinguishable from the controls (Figures 2.32A and 2.32B). A small amount of conidiation was observed on the mycelial plugs at the lower pH values. All cultures had a 'wet' appearance, which was not observed in the PDB photoconidiation or the pH-buffered PDYC experiments.

Unbuffered PDB

In the control, sparse conidiation was present on the mycelial plugs at all pH values, though barely discernible at higher pH (Figure 2.33A). The 'wet' appearance was observed from pH 2.8 to 3.6 only. Similarly, sparse conidiation was also present on the mycelial plugs from the treatment plates and the wet appearance persisted from pH 2.8 to 3.6 (Figure 2.33B). As observed on PDYC, conidiation in response to light was observed from pH 4.0 to 5.2. Two immature rings of conidia were produced rather than a single mature ring and the outer ring represented the position of the colony front at the time of light exposure. When plates were incubated for longer, conidiation matured. Photo-induced conidiation was also observed in the first PDB assay (2.15.1.2) and in the unbuffered PDYC experiment from pH 4.0 to 5.2, however in both experiments a single ring of green conidia was produced.

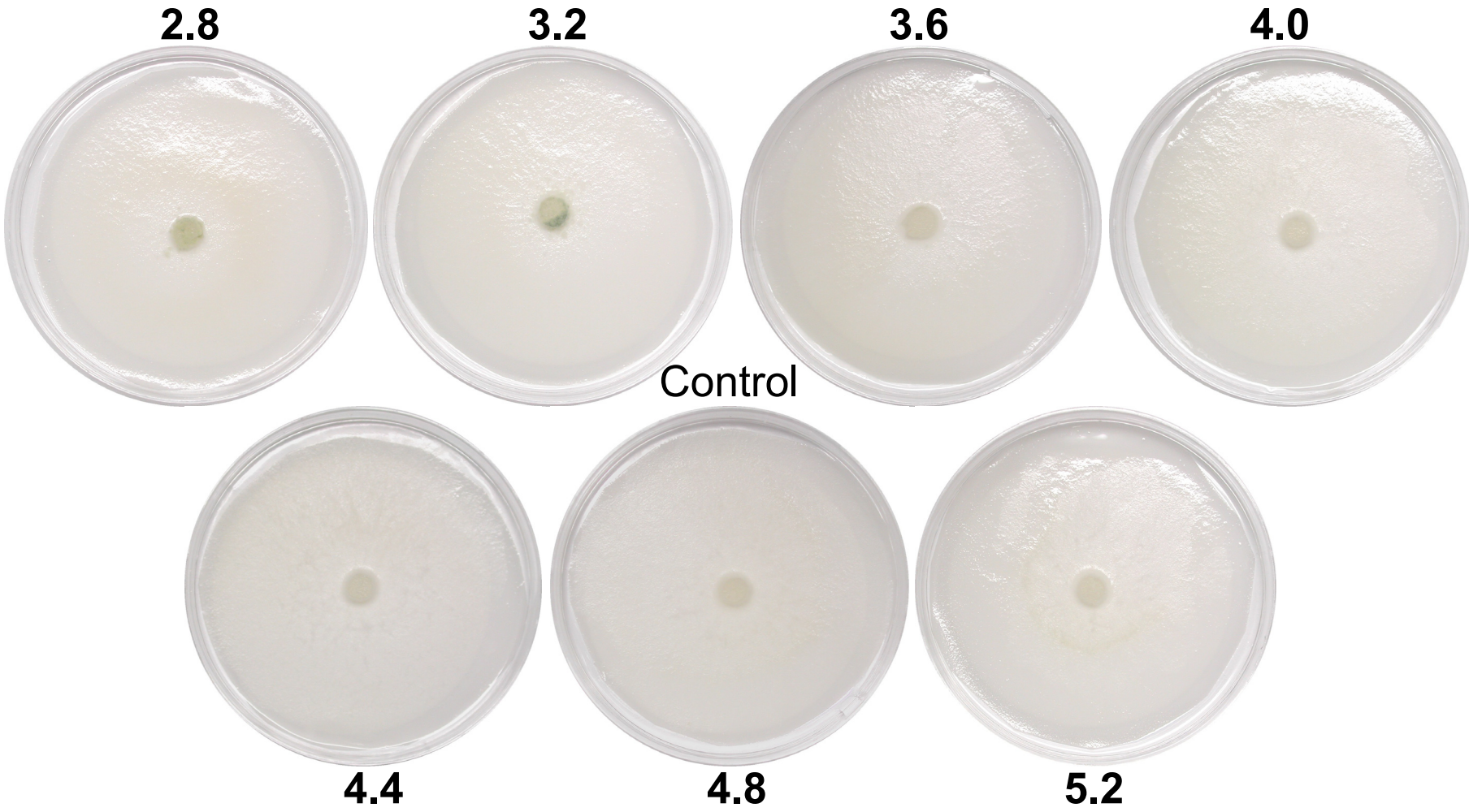


Figure 2.32A. *Trichoderma atroviride* photoconidiation experiment (control plates) on pH buffered (2.8 to 5.2) PDB-soaked filter paper. Cultures were grown for 4.75 d (114 h) in total darkness.

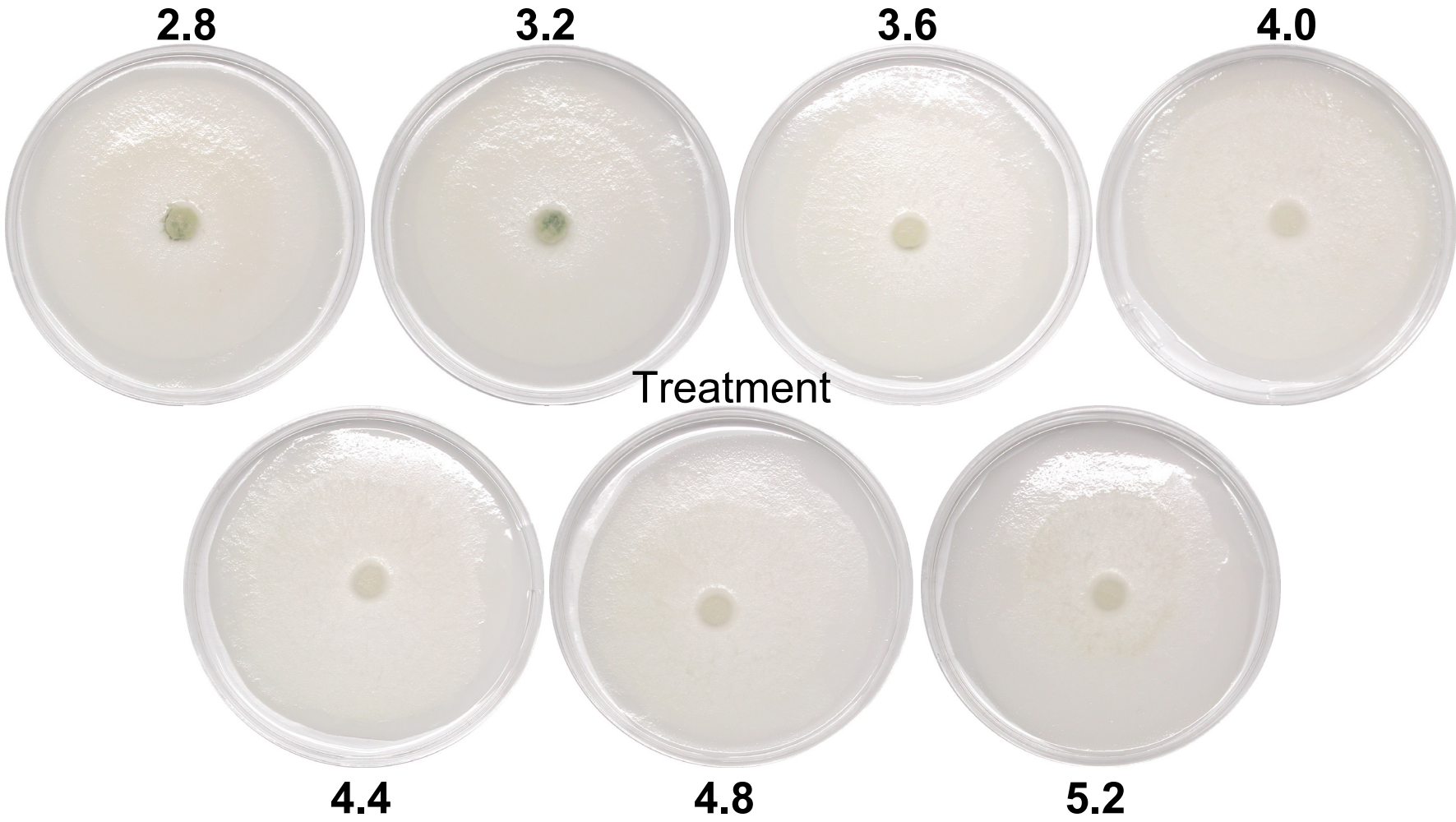


Figure 2.32B. *Trichoderma atroviride* photoconidiation experiment (treatment plates) on pH buffered (2.8 to 5.2) PDB-soaked filter paper. Cultures were grown for 42 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

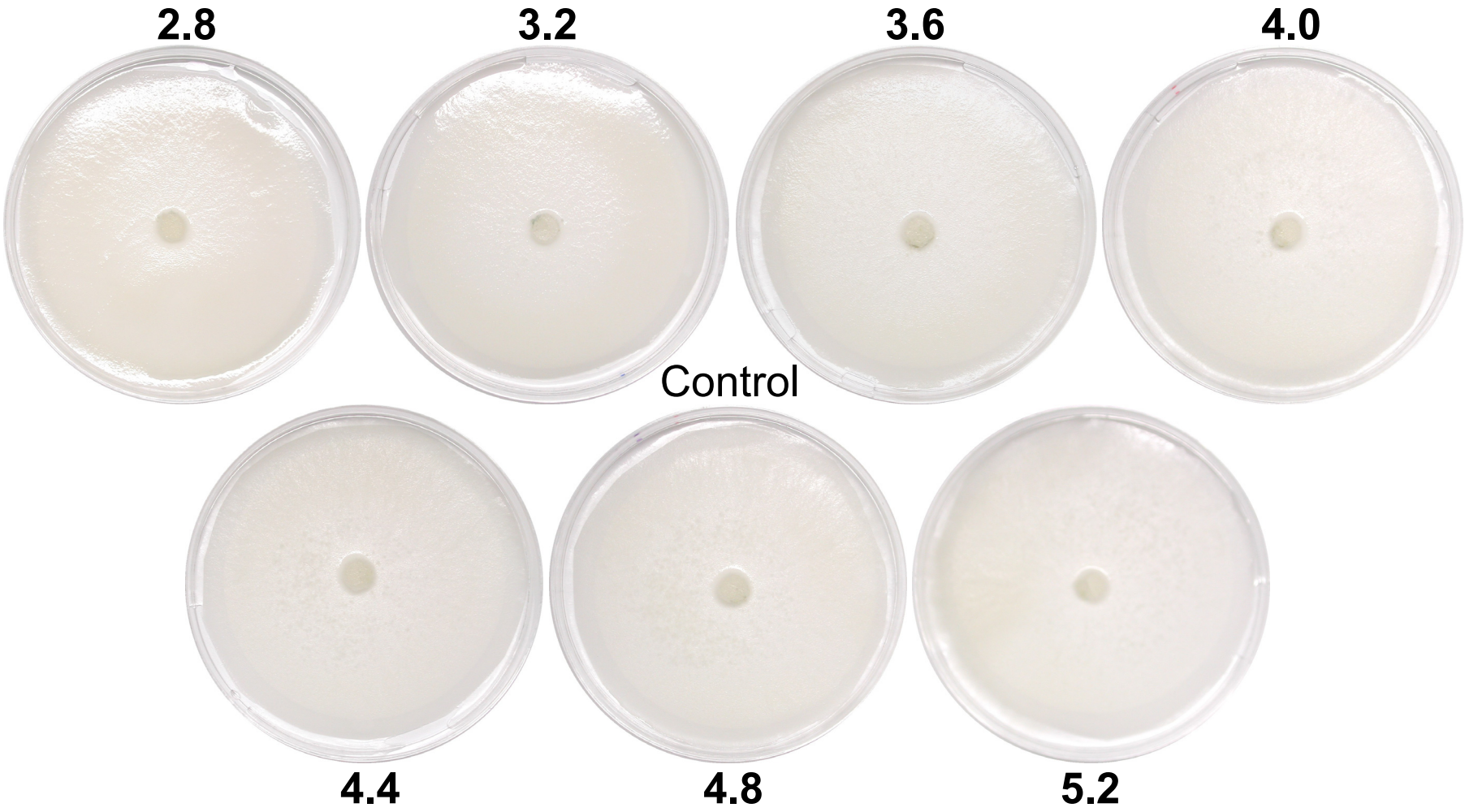


Figure 2.33A. *Trichoderma atroviride* photoconidiation experiment (control plates) on unbuffered, pH adjusted (2.8 to 5.2) PDB-soaked filter paper. Cultures were grown for 4.75 d (114 h) in total darkness.

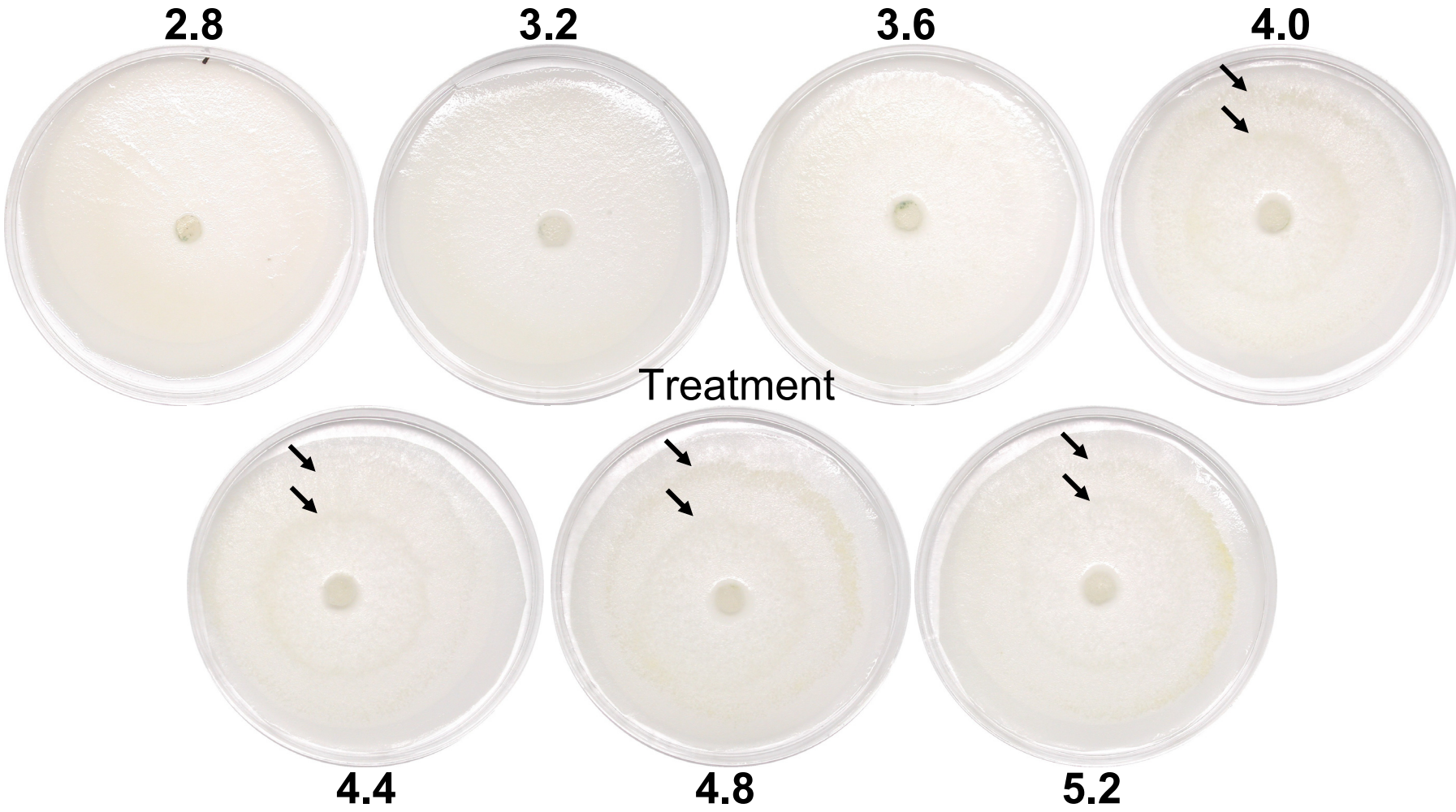


Figure 2.33B. *Trichoderma atroviride* photoconidiation experiment (treatment plates) on unbuffered, pH adjusted (2.8 to 5.2) PDB-soaked filter paper. Cultures were grown for 42 h in total darkness, then photoinduced and grown a further 72 h in total darkness. The arrows point to the immature rings of conidia.

2.15.3.2 *Trichoderma harzianum*

pH-buffered PDB

In contrast to the first PDB photoconidiation experiment (2.15.1.2), the pH-buffered PDYC experiment (2.15.2.2) and the PDA experiment (2.11.5), conidiation in response to light was barely discernible in *T. harzianum* on the pH-buffered PDB-soaked filter paper and treatment plates were virtually indistinguishable from the controls. Green conidia were observed on and next to the mycelial plugs of the pH 2.8 to 4.4 control plates (Figure 2.34A) and this was a little more intense on the treatment plates (Figure 2.34B). This differed from the pH-buffered PDYC experiment, in which conidiation was observed at all pH values. All cultures had a 'wet' appearance as described for the *T. atroviride* pH-buffered PDB plates. From pH 4.4 to 5.2 a yellow pigment was observed at the centre of the plates and this was most intense at pH 4.8 to 5.2. This differed from previous *T. harzianum* pH-based experiments in which the yellow pigment did not appear until pH 4.8, with the exception of the unbuffered, pH adjusted PDYC experiment (2.15.2.2).

Unbuffered PDB

In contrast to the pH-buffered PDB plates and similar to the unbuffered PDYC experiment, mature conidia were observed on all control plates, though conidiation was noticeably less intense than on PDYC. The 'wet' appearance of the colony described for the pH-buffered PDB filters was also observed on the unbuffered PDB at all pH values. This differed from the unbuffered PDYC experiment (2.15.2.2) in which the wet appearance was observed from pH 2.8 to 3.6 only. At pH 2.8, a few tufts of conidia were produced on and beside the mycelial plug on the control plates (Figure 2.35A). More conidia were observed on the pH 3.2 controls and they were diffusely distributed across one third of the plate. At pH 3.6, dense conidiation was observed mainly around the mycelial plug and a yellow pigment was faintly present on the filter paper. This differed from all previous pH experiments in which the pigment first appeared at pH 4.4 or 4.8. Pigment production became more intense as the pH rose. At pH 4.0, conidiation was on and round the mycelial plug arranged in a small ring, similar to the unbuffered pH 4.0 PDYC control plate. Similarly, a small ring was observed on the pH 4.4 and 4.8 plates. In addition, banding of the yellow pigment was observed on the pH 4.8 filter papers. At pH 5.2, the banding was absent, however, there was a zone free of pigment at the centre of the plate in which sparse conidiation was observed similar to the first PDB experiment (2.15.1.2). In contrast, the yellow pigment was produced in the central zone

of the pH-buffered PDB pH 4.8 to 5.2 plates and the rest of the plate was free of pigment.

At all pH values there was an increase in conidiation in response to light, however conidiation correlated with the colony margin at the time of exposure only at pH 3.2 and 3.6. At pH 2.8 a small sparse ring of green conidia was observed at the centre of the plate (Figure 2.35B), which differed from the unbuffered PDYC where a much larger ring was observed and the pH-buffered PDA experiments where a thick ring of conidia were produced. At pH 3.2, a thick diffuse ring of conidia was observed and this was more intense at 3.6. The margins of both disks correlated with the colony margin at the time of light exposure. An additional thin green ring of conidia was observed at both pH 3.6 and pH 4.0, and this arose from hyphae which formed after the light exposure. From pH 4.4 to 5.2 conidiation intensified compared with the controls but was constrained to the centre of the plates. The yellow pigment was observed from pH 4.0 to 5.2 and this was identical in distribution and intensity to the control plates. Conidiation was observed within the central pigment-free zone at the pH 4.8 and 5.2. At pH 5.2 sparse conidiation was observed beyond this central zone and closer examination of the treatment plates showed this correlated with a reduction in the amount of pigment impregnated in the filter. The pH 5.2 plates closely resembled the PDB plates from 2.15.1.2.

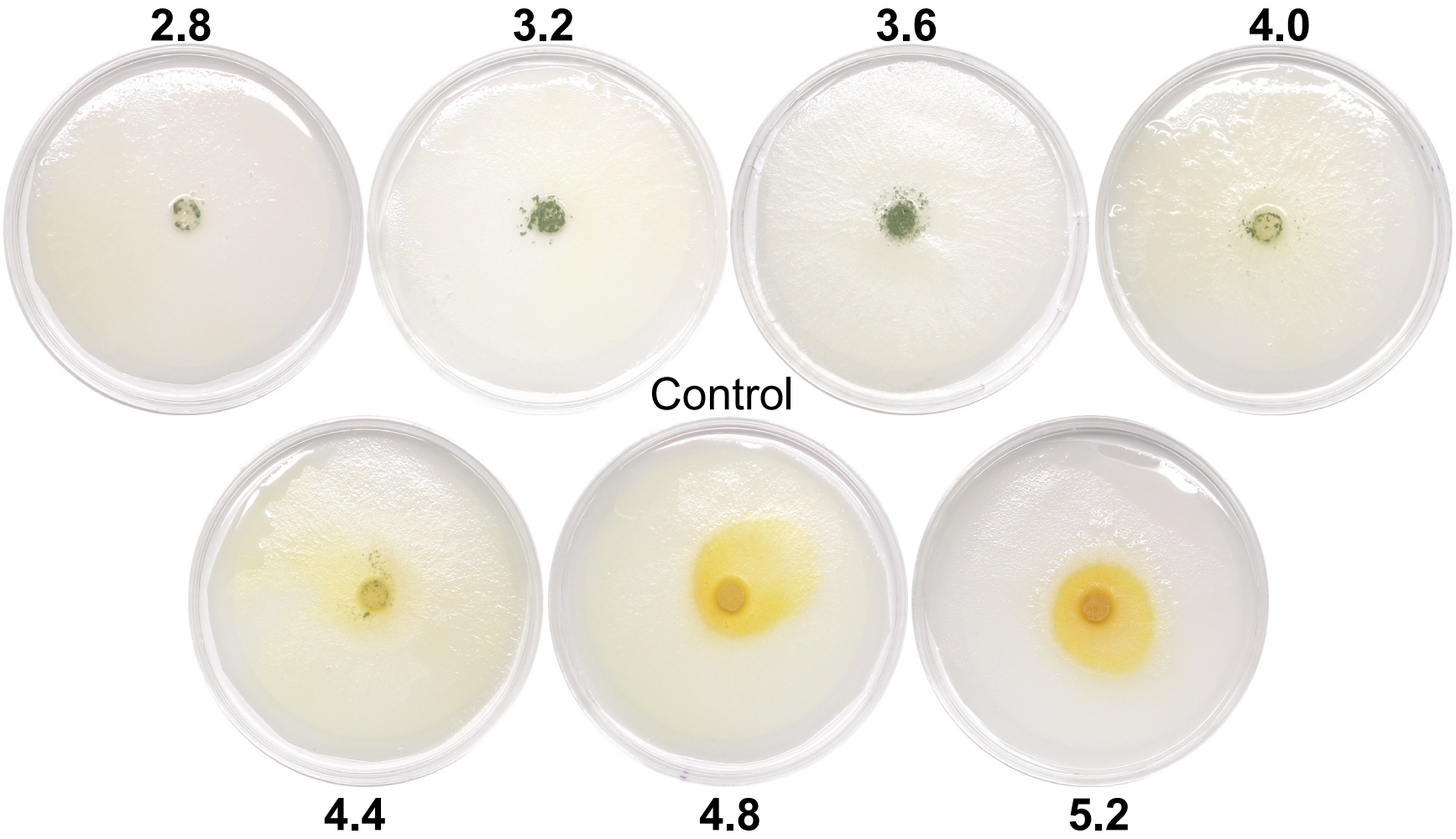


Figure 2.34A. *Trichoderma harzianum* photoconidiation experiment (control plates) on pH-buffered (2.8 to 5.2) PDB-soaked filter paper. Cultures were grown for 4.75 d (114 h) in total darkness.

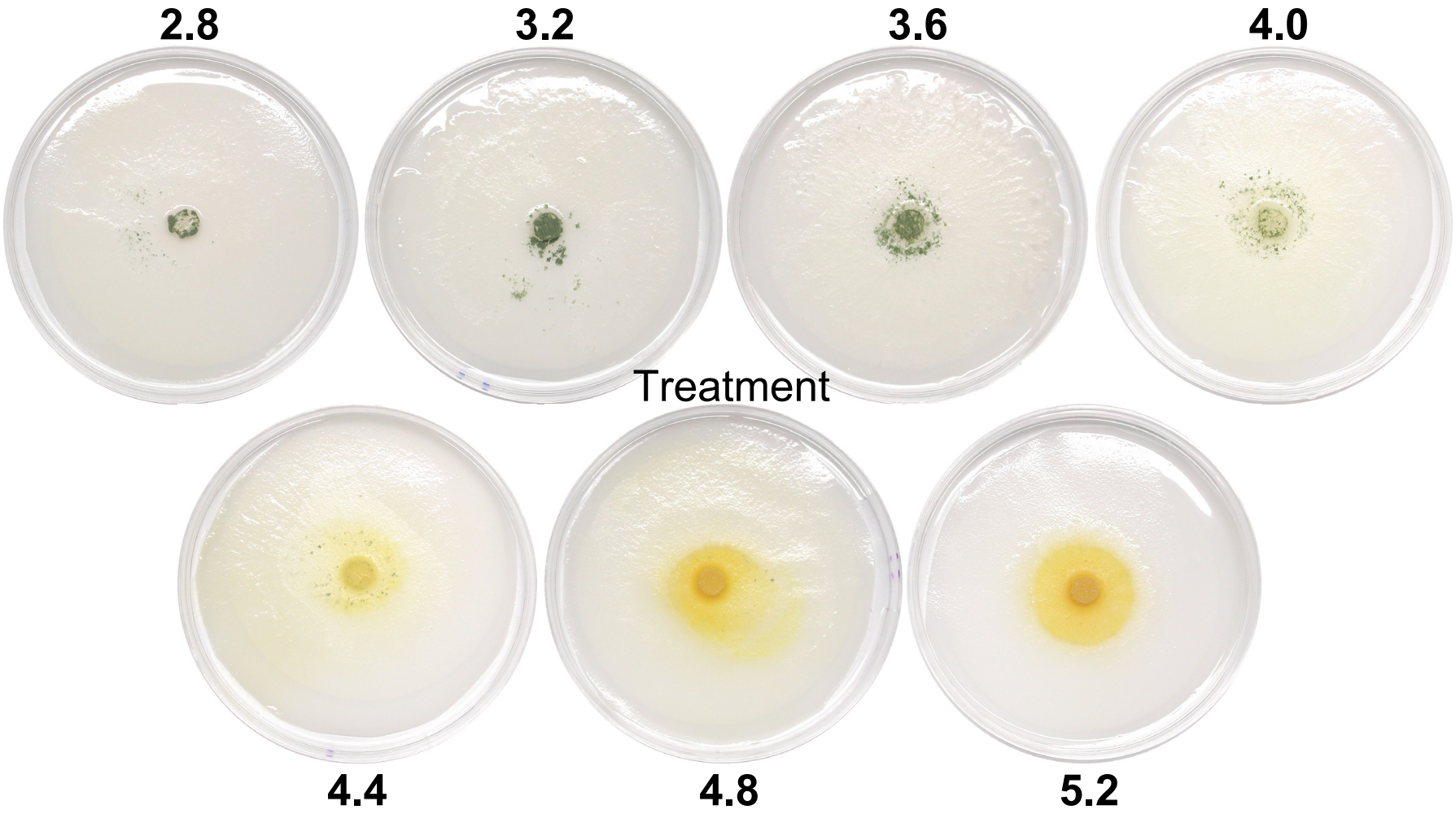


Figure 2.34B. *Trichoderma harzianum* photoconidiation experiment (treatment plates) on pH-buffered (2.8 to 5.2) PDB-soaked filter paper. Cultures were grown for 42 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

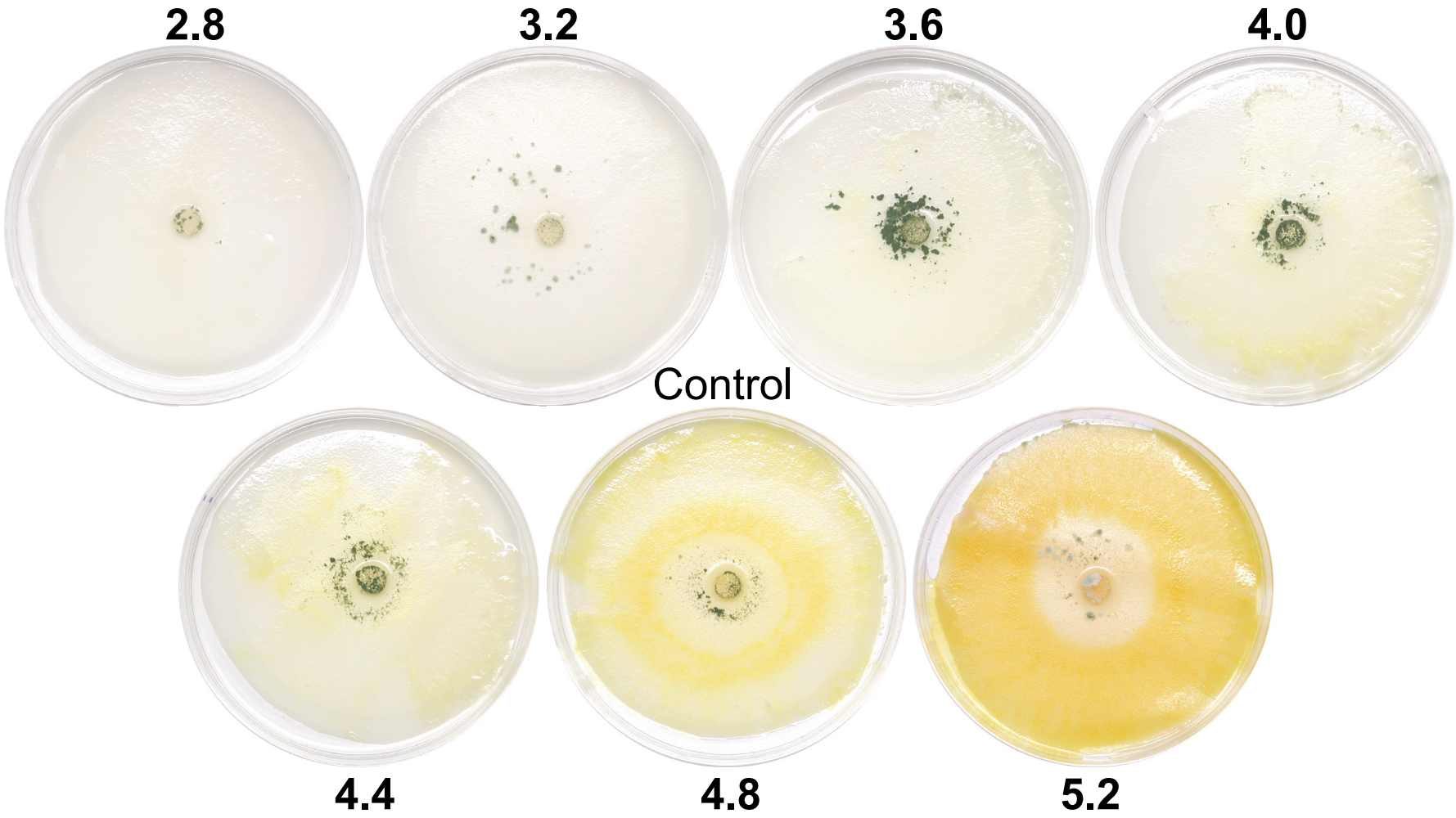


Figure 2.35A. *Trichoderma harzianum* photoconidiation experiment (control plates) on unbuffered, pH adjusted (2.8 to 5.2) PDB-soaked filter paper. Cultures were grown for 4.75 d (114 h) in total darkness.

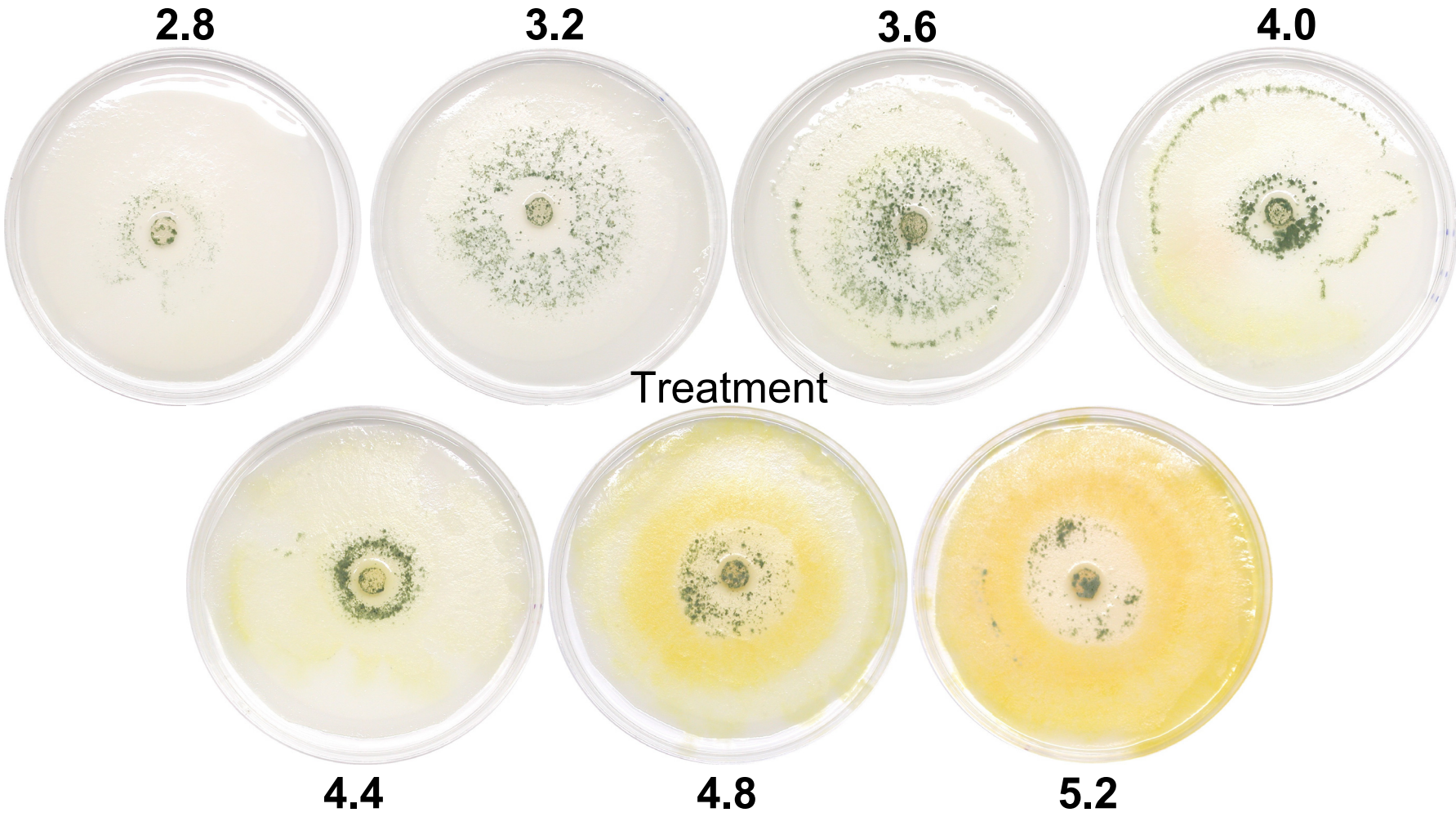


Figure 2.35B. *Trichoderma harzianum* photoconidiation experiment (treatment plates) on unbuffered, pH adjusted (2.8 to 5.2) PDB-soaked filter paper. Cultures were grown for 42 h in total darkness, then photoinduced and grown a further 72 h in total darkness.

2.16 Discussion

2.16.1 Broth-Soaked Filter Paper Promotes Photoconidiation in a Ring in *T. hamatum*

On broth-soaked filter paper, *T. hamatum* photoconidiated in a clear ring which correlated with the colony perimeter, whereas in Sections A and C, this isolate responded weakly to a single light dose producing a sparse cluster of conidia at the centre of the agar plates. This suggests that some aspect of the filter paper assay strongly promoted photoconidiation in a ring in *T. hamatum*. In the previous sections, differences in the nutrient status of the medium have been demonstrated to have a significant impact on conidiation and this varied between species/isolates. The reduction in medium volume in the filter paper assay would have reduced the total nutrients available by approximately 90% and, therefore, by comparison, the assay was conducted at a lower nutrient status than on agar. It is possible that photoconidiation in *T. hamatum* is favoured in a ring under conditions of low nutrients. It is also equally possible that the differential photoconidial response may be due to the use of filter paper as the support substrate. Both could be tested by assaying photoconidiation on 2-3 mL PDA. If the appearance of a ring in *T. hamatum* relates directly to nutrient limitation, then photoconidiation on thin PDA should resemble that on PDB-soaked filter paper.

In response to a single light-dose, both rings and disks of conidia have been observed in this study for four out of five isolates of *Trichoderma* representing different species and this was dependent on the nutritional status and experimental design. This study has clearly demonstrated that conidiation can occur at the periphery of the colony, at the centre and gradients in between. It is likely that multiple environmental factors affect photoconidiation patterning and from these studies it is evident that under a given set of conditions, photoconidial patterning is isolate-specific. The mechanism(s) behind the variation has not been determined, however, on the basis of the experiments presented in this chapter, I postulate that variation along the spectrum of photoconidiation relates to an individual's ability to uptake and assimilate nutrients under variable environmental conditions.

2.16.2 The Influence of Ambient pH on Photoconidiation is Dependent on the Environment

On buffered PDYC and PDB-soaked filter paper, no conidiation in response to light was observed in *T. atroviride*, whereas photoconidiation was observed on unbuffered

medium, which suggests that buffering of the medium inhibited photoconidiation on filter paper in this isolate. In stark contrast, photoconidiation was strongly induced on buffered PDA and, in addition, was strictly low pH-dependent (2.11.1.1). Clearly some aspect of the filter paper assay design negatively influences the ability of *T. atroviride* to photoconidiate under buffered conditions. In *T. harzianum*, photoconidiation was promoted by low pH on broth-soaked filter paper in a similar fashion to that observed in Section C on PDA, which suggests that unlike *T. atroviride* the use of the filter paper assay does not affect the influence of ambient pH on photoconidiation in *T. harzianum* LU675.

Conidiation in response to light was observed on unbuffered PDYC and PDB and this occurred in a pH-dependent fashion, however, unlike that observed on unbuffered PDA (2.11.1.1) no conidiation was observed below pH 4.0. In Section C, it was hypothesised that low pH promotion of photoconidiation on buffered PDA was related to intracellular acidification and/or the promotion of nutrient uptake by low ambient pH. The absence of conidiation below pH 4.0 clearly questions these hypotheses and suggests that the relationship between ambient pH and the environment is much more complex than previously thought. It would appear from the studies presented in this and previous sections that the influence of ambient pH on photoconidiation in *T. atroviride* is determined by other aspects of the environment. As suggested for the buffered experiments above, some aspect of the filter paper assay likely affected the photoconidial phenotype and this may be nutrient limitation or the filter paper itself. Assessment of photoconidiation from pH 2.8 to 5.2 on 2-3 mL unbuffered PDYC agar or PDA would test this theory. If nutrient limitation was the cause of the shift in influence of ambient pH then photoconidiation on thin agar should resemble that on broth-soaked filter paper.

On unbuffered PDB, the conidial morphology was altered in *T. atroviride* compared with previous filter paper and agar assays on PDB or PDA. Two rings of immature conidia were produced in response to light, whereas on standard PDB (2.15.4.2) and unbuffered PDYC from pH 4.0 to 5.2, a single ring of green conidia was formed. This change in morphology was unexpected and suggested that some unknown variable which influenced photoconidiation had entered the experiment. Two rings were also observed when the experiment was repeated, however when the replicates were increased significantly some plates produced a single green ring and some two rings

(data not shown). This observation resulted in a series of experiments to determine the cause of the variation, however nothing conclusive was able to be drawn (Appendix 7.3.2 to 7.3.7). Unexplained variation has been observed previously in isolates of *T. viride* (Nemcovic & Farkaš, 1998). These authors described an apparent seasonal variation in the reproducibility of filter paper assays. Using the same isolate Gresik *et al.* (1991) reported that, for a good photoconidial response, the mycelium needed to be in good contact with the air and no residual moisture should be present on the surface of the filter paper. In the experiments presented here all the PDB plates had a wet appearance, however this was absent when larger plates and filters were used and, in addition, conidiation always occurred in a single ring on the larger plates (Appendix 7.3.7, Figure 7.9).

2.16.3 PDYC Promotes Photoconidiation Relative to PDB

The addition of yeast extract and casein hydrolysate promoted photoconidiation in four of the five isolates in this study and in *T. harzianum*, it strongly promoted conidiation in the dark. Yeast extract is a complex of vitamins and nutrients and casein hydrolysate is a rich source of amino nitrogen. Either supplement may have promoted photoconidiation. It is not known whether yeast extract alone promotes the conidial response, however casein hydrolysate is a rich source of primary nitrogen and primary nitrogen has been postulated in this study to promote photoconidiation in *Trichoderma*. It is possible that the promotion of photoconidiation on PDYC compared to PDB is due to the effect of extra primary nitrogen. This could be tested by repeating the pH filter assays using yeast extract and casein hydrolysate alone and in combination to determine if casein hydrolysate has a major role. To test for nitrogen involvement directly, alternative primary nitrogen sources could be added at equimolar amounts.

2.16.4 Pigment Production is Altered in Response to Ambient pH on Filter Paper in *T. harzianum*

Alterations in the excretion of the yellow pigment in response to the ambient pH were observed on filter paper in comparison with PDA. On buffered PDB-soaked filter paper, the yellow pigment was first observed at pH 4.0 and on unbuffered PDB at pH 3.6, whereas in Section C, the pigment did not appear until pH 4.8 and above on PDA. These results suggest that an aspect of the filter paper assay influenced pigment production in this isolate. Interestingly, when yeast extract and casein hydrolysate were added to the medium (PDYC), the pigment was not observed till pH 4.8 on buffered

medium and 4.4 on the unbuffered medium, which suggests that addition of these supplements ameliorated the effect of the filter paper assay on early pigment production.

On unbuffered filter papers, there was a circular zone in the centre, in which little or no pigment was observed. This clear zone was sharply delineated by profuse impregnation of the filter paper with the yellow pigment and was noticeably larger on PDB-soaked filter paper compared with PDYC, which suggests that there is a delay before pigment production/excretion occurs. In sharp contrast, the yellow pigment was clearly observed at the centre of the plates on buffered PDB and the pigment was constrained to the centre only, which suggests that buffering of the medium suppresses the delay in pigment production.

Like conidiation, production and excretion of the yellow pigment may be rhythmic under some circumstances. At pH 4.8, on unbuffered PDB, pigmentation appeared in bands on the filter paper. In addition, conidiation at the centre of both the PDYC and PDB-soaked filters was constrained within the central clear zone and photoconidiation appeared to alternate with regions of high pigment production. In the pH assays it would appear that conditions which promote pigment production do not favour conidiation and vice versa, which suggests that pigment production and conidiation may be temporally separated due to different intracellular metabolic requirements.

A yellow pigment associated with conidiation has been observed previously in photoconidiation mutants of *T. atroviride*. Horwitz *et al.* (1985a) isolated two mutants (*dimY*)., which were photoconidiation defective, yet conidiated well in response to nutrient starvation and produced a yellow pigment into the medium The authors postulated this pigment to be a precursor for a photoreceptor, however further work demonstrated the *dimY* mutation(s) not to be involved with the *blr-1* and *blr-2* loci (Casas-Flores *et al.*, 2004; Section 1.5.3). The authors did not identify the pigment, however given the apparent relationship of this pigment to conidiation, it is possible that their pigment and the yellow pigment observed in this study are the same. Identification of the yellow pigment will significantly aid in interpretation of these results.

Chapter Three
Analysis of Circadian Rhythms

3.1 Introduction

In response to cyclical changes in light, temperature and other environmental influences, organisms have adapted to anticipate daily changes through the evolution of circadian rhythms (Lakin-Thomas and Brody, 2004). These rhythms have been well characterised in the blue-light fungus *Neurospora crassa*, which conidiates in the dark in a circadian fashion following exposure to light. Betina and Zajacová (1978) and Schrüfer and Lysek (1990) investigated whether *Trichoderma* cultures could also conidiate rhythmically following a dose of light and concluded that no circadian rhythm was apparent and conidiation was strictly light-inducible. Deitzer *et al.* (1988) investigated circadian rhythms in *T. atroviride* (formerly *T. harzianum* [Kindermann *et al.*, 1988]) (Section 1.4.2.3). Though no rhythmic conidiation occurred in the dark, a subtle 24 h oscillation in light sensitivity was observed in dark-grown *T. atroviride* cultures, as measured by light-induced conidiation. Rhythmic conidiation in the dark was, however, observed in a mutant derivative (Deitzer *et al.*, 1988). Under constant darkness the mutant produced well-defined concentric rings about 12 h apart and a light pulse resulted in a shifting of the banding pattern. The addition of deoxycholate to the medium slowed the growth rate of both the wild-type and the mutant and caused the wild-type to conidiate in rings under constant light. The rings were produced with circadian periodicity and the period of the rings produced by the mutant under constant darkness became circadian-like also. The use of this detergent to increase the period length in the mutant and the observation of both the light sensitivity and banding pattern in dark-grown cultures suggested these endogenous rhythms were not circadian or light induced (Deitzer *et al.*, 1988). The authors also raise the possibility that deoxycholate may have an affect on circadian rhythmicity.

In Chapter 2 of this study, rhythmic conidiation was observed in *T. atroviride* and *T. harzianum* in five separate experiments (Figure 3.1). On PDA buffered to pH 2.8, *T. atroviride* cultures of sufficient age responded to light by producing conidia which appeared to cluster into two rings. This occurred in both the conidial and mycelial plug inoculum experiments (Sections 2.11.3.1 & 2.11.3.2). On PDA buffered from 2.8 to 3.2, a second ring-like arrangement of conidia was observed in *T. harzianum* cultures

beyond the light-induced ring and this arose from hyphae not yet formed at the time of light exposure (Section 2.11.5.1). When a mycelial plug was used as inoculum, a ring of conidia was produced on the control in the absence of light at pH 2.8 to 3.2 (Section 2.11.5.2). On PDYC-soaked filter paper, a ring of conidia was observed at the centre of the plates on both the dark controls and light treatment plates, and conidiation was also observed at the edge of the filter paper (Section 2.15.1.1). A possible explanation for all of these observations is rhythmic conidiation.

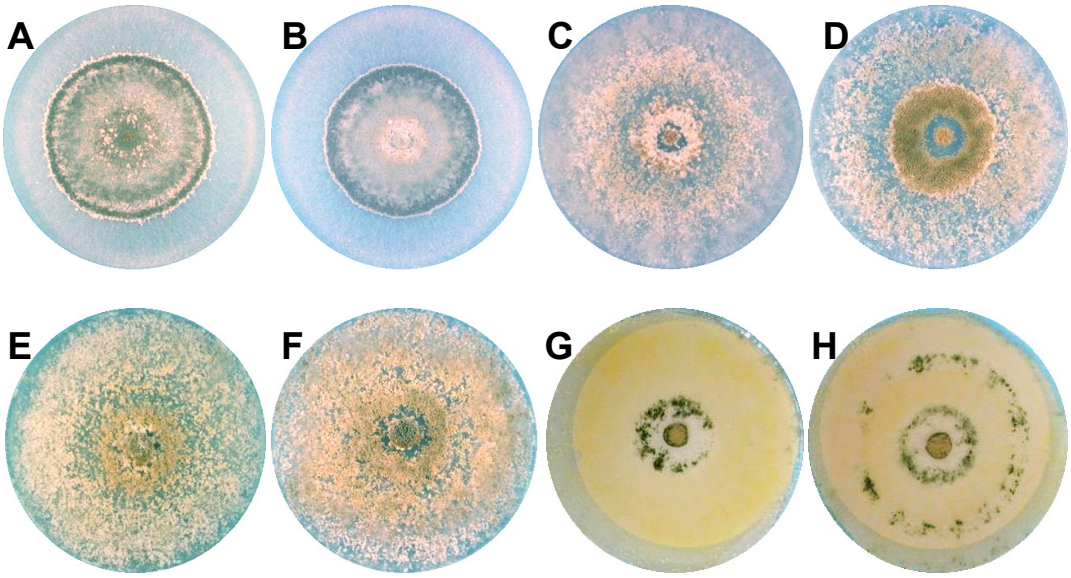


Figure 3.1. Rhythmic conidiation observed in photoconidiation experiments presented in Chapter 2 of this study**. A-B.** *T. atroviride* on PDA buffered to 2.8. **A.** The plate was inoculated with conidia and light given at 72 h (Section 2.11.3.1, Figure 2.19B). **B.** The plate was inoculated with mycelial plugs and light given at 48 h (Section 2.11.3.2, Figure 2.20B). **C-F.** *T. harzianum* on PDA buffered from 2.8 **(C, E)** to 3.2 **(D, F)**. **C-D.** Plates were inoculated with conidia and light given at 48 h (Section 2.11.5.1, Figure 2.22B). **E-F.** Plates were inoculated with mycelial plugs and grown in total darkness (Section 2.11.5.2, Figure 2.23A). **G-H.** *T. harzianum* on PDYC-soaked filter paper. Plates were inoculated with mycelial plugs and incubated in total darkness **(G)** or light was given at 36 h **(H)** (Section 2.15.1.1, Figure 2.26).

Multiple rings of conidia were most clearly observed in *T. harzianum* LU675 on PDYC-soaked filter paper, therefore this isolate and system was chosen to investigate rhythmic conidiation. To extend the time before cultures reached the edge of the plate, extra large filters and plates were used. It was postulated that in response to a single light exposure, *T. harzianum* would conidiate in a rhythmic fashion in the modified PDYC-soaked filter paper assay.

Race tube analysis is commonly used to investigate rhythmic conidiation in *N. crassa* and has more recently been used to demonstrate rhythmic sclerotial production in *Aspergillus flavus* (Loros & Dunlap, 2001; Greene *et al.*, 2003). The design of the race tube results in elevated levels of CO_2, which, in *N. crassa*, prevents conidial formation in response to light. To overcome this problem, most laboratories use a *N. crassa* strain carrying a mutation in the *band* gene (Sargent *et al.*, 1966), which alleviates the inhibitory effects of CO_2 (Loros & Dunlap, 2001). The effects of CO_2 on photoconidiation in *Trichoderma* spp. are not known, however an absence of oxygen does inhibit development of conidia in response to light in *T. viride* (Gutter, 1957; Gressel *et al.*, 1975) and in this study it has been observed that sealing of the plates significantly reduced conidiation (data not shown). It was postulated that conidiation in race tubes would be negatively affected in *T. harzianum* as it is in *N. crassa*, therefore race tubes were not used and instead large Petri dishes and filters were employed.

In *N. crassa*, the WC-1 and WC-2 proteins, together with FRQ (Frequency) are the key regulators of circadian rhythms, where WC-1 and WC-2 regulate gene expression and FRQ acts as the oscillator (Loros & Dunlap, 2001; Lakin-Thomas & Brody, 2004). This circadian clock has been the subject of much research, however it is evident from the literature that a considerable amount is still unknown. Rhythms which do not require the WC proteins but respond to blue-light have been observed, suggesting additional blue-light receptors. Endogenous daily rhythms linked to metabolism, which oscillate independently of FRQ, have also been observed, such as the nitrate reductase rhythm (Christensen *et al.*, 2004). Orthologues of the WC proteins, BLR-1 and BLR-2, have recently been identified in *T. atroviride* and shown to mediate blue-light induced photoconidiation (Casas-Flores *et al.*, 2004, Section 1.5.1). *blr-1* and *blr-2* orthologues have also been published from the *T. reesei* genome (ascn#: AY823264 and AY823265). It is not known whether BLR-1 and BLR-2 are involved in circadian responses in *Trichoderma* spp. and though they are highly similar, BLR-1 is unable to complement WC-1 in *N. crassa* (Alfredo Herrera-Estella, pers. comm.). Orthologues of the *N. crassa frq* gene have been identified in a number of filamentous fungi (Lombardi & Brody, 2005), however to the best of our knowledge none have been identified in *Trichoderma.* The second aim of this chapter is to determine whether orthologues of *blr-1* and *frq* are present in the *T. atroviride*, *T. hamatum* and *T. harzianum* isolates under consideration in this study. It was hypothesised that gene orthologues of the

components of the WCC/FRQ clock from *N. crassa* would be present in all *Trichoderma* spp. isolates examined and would also exhibit a high degree of similarity.

A number of genes, which are under the influence of the WC/FRQ clock, have been identified in *N. crassa* and demonstrated to be expressed in a rhythmic fashion under constant conditions. Rhythmic gene expression of clock-controlled genes (*ccg*) is typically analysed using the free-running rhythm (FRR) assay (Loros & Dunlap, 2001; 1991). In brief, liquid-grown cultures are exposed to light for 12 h then transferred to constant darkness for 12 h and sampled every 4 h thereafter for RNA extraction. In both *N. crassa* and *A. nidulans*, the FRR assay has been used to demonstrate rhythmic expression of the *gpd* gene (*ccg-7*), which encodes the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Shinohara *et al.*, 1998; Greene *et al.*, 2003). In *T. atroviride*, *gpd* is significantly down-regulated within 16 h post light exposure (Baum & Horwitz, 1991; Puyesky *et al*, 1997), which clearly shows that *gpd* expression is affected by light in *Trichoderma*, however it is not known whether *gpd* exhibits a rhythm. In this chapter, the FRR assay was used to investigate *gpd* expression in *T. atroviride* IMI206040 and a *blr-2* knockout mutant, which is unable to conidiate in response to light (Alfredo Herrera-Estrella, pers. comm.; Casas-Flores *et al.*, 2004). It was hypothesised that expression of *gpd* in *T. atroviride* exhibits a free-running rhythm under constant conditions and that this rhythm is associated with a functional BLR complex.

3.2 Materials and Methods

3.2.1 Fungal Isolates

Three *Trichoderma* biocontrol isolates from New Zealand and two from Mexico were used in this study. The New Zealand isolates were obtained from the Lincoln University culture collection and included *T. atroviride* LU298, *T. hamatum* LU592 and *T. harzianum* LU675. Isolate origins are presented in Table 2.1, Section 2.2.1. Two *T. atroviride* isolates, IMI206040 and a derivative with mutations in the *blr-2* gene, were kindly donated by Professor Alfredo Herrera-Estrella (Unidad Irapuato) for use in this study. The mutant *Δblr-2* was constructed by replacement of the *blr-2* gene with the *E. coli hph* gene, which encodes hygromycin phosphotransferase, under the control of the *glaA* promoter from *Aspergillus niger* (Casas-Flores *et al.*, 2004). Isolates were maintained and stored on potato-dextrose agar (PDA) (Appendix 7.2.1.).

3.2.2 Analysis of Rhythmic Conidial Production in *T. harzianum*

The phenomenon of circadian rhythm-associated conidiation was investigated in *T. harzianum* isolate LU675 using a modification of the filter paper photoconidiation assay. Mycelial plugs from 2 day old cultures were inoculated to three 120 mm Whatman #1 filters, which had been soaked in PDYC and placed in 150 mm Petri dishes. Plates were incubated unsealed at 23°C in total darkness for 48 h, then exposed to blue light for 15 min, as described in 2.2.3 and then incubated for a further 96 h in total darkness. Control plates were incubated in total darkness for the duration of the experiment. Four plates per treatment were inoculated and the experiment was repeated once.

3.2.3 Extraction of Genomic DNA for use in PCR Amplifications

Genomic DNA was isolated from all isolates (3.2.1) using the Puregene® Genomic DNA Purification Kit (Gentra Systems Inc., Minneapolis, Minnesota, USA) as per manufacturer's instructions. Approximately 200 mg of frozen mycelium (Appendix 7.2.4) was placed in a sterile mortar and pestle containing liquid nitrogen. The mycelium was ground to a fine powder and 20 mg transferred to a chilled centrifuge tube containing 600 µL of cell lysis buffer, 30 µg RNaseA and 60 µg Proteinase K (Roche Diagnostics GmbH., Mannheim, Germany). The mixture was immediately homogenised using a wide-bore pipette tip, and placed on ice. Samples were incubated at 55°C for 60 min, with inversion of tubes once. Tubes were cooled to room temperature and 200 µL protein precipitation buffer added. Tubes were inverted 10 times and placed on ice for 15 min. The solution was centrifuged at 20 000 x*g* for 5 min and the supernatant containing DNA transferred to a fresh tube. DNA was precipitated by addition of one volume of 100% isopropanol and incubation on ice for 5 min, then pelleted by centrifugation at 20 000 x*g* for 10 min at 4°C. The pellet was washed twice in 70% ethanol, air-dried, resuspended in 50 µL of sterile water and stored at 4°C. DNA samples were quantified via gel electrophoresis as described in Appendix 7.2.5.

3.2.4 Isolation of the *blr-1* Gene

3.2.4.1 Amplification of *blr-1*

Using the published (http://www.ncbi.nlm.gov/Genbank) *blr-1* sequences from *T. atroviride* (ascn#: AY628431) and *T. reesei* (ascn#: AY823264) primers were designed to amplify a 750 bp region from 1040 bp 3' to the translational start point of *blr-1* from *T. atroviride* LU298, *T. hamatum* LU592 and *T. harzianum* LU675. Primers blr1-F (5'-

CGAYATGTCCTGTGCCTTTG-3') and blr1-R (5'-GTTGCGTCTTTCAGCTCCC-3') were designed to regions of 99.9% identity between *T. atroviride* and *T. reesei blr-1*. All PCR amplifications were performed in a Bio-Rad Icycler™ (Bio-Rad Laboratories, Hercules CA, USA). Each 25 µL PCR reaction contained 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM MgCl2, 200 µM each of dATP, dCTP, dGTP and dTTP (Roche Diagnostics GmbH.), 10 pmoles of each primer, 10 ng of LU298, LU592 or LU675 gDNA and 1.25 U Taq DNA polymerase (Roche Diagnostics GmbH.). To check for contamination, a negative control was included that contained all the above reagents except template DNA. Amplification consisted of an initial denaturation of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C, followed by a final extension of 10 min at 72°C. Resulting PCR products were size fractionated by 1% gel electrophoresis as described in Appendix 7.2.5.

3.2.4.2 TA Cloning of PCR Products

PCR products obtained as described above were ligated into the TA-cloning site in pGEM®-T (Promega Corp., Madison WI, USA) (Appendix 7.5.1) as per manufacturer's instructions and transformed into *E. coli* strain INVαF' (Invitrogen Corp., Carlsbad, CA, USA) using standard techniques (Sambrook *et al.*, 1989). Typically, a 2 µL aliquot from the ligation reaction was mixed with 40 µL of competent cells and incubated on ice for 30 min. Cells were heat shocked at 42°C for 40 s to facilitate DNA uptake, then placed on ice for 2 min. A 350 µL aliquot of SOC medium (Appendix 7.2.6) was added and transformations were incubated at 37°C for 1 h to allow expression of the ampicillin resistance gene. A 100 µL aliquot of the transformation mix was spread on LB/Amp/X-Gal selection plates (Appendix 7.2.6) and incubated overnight at 37°C. Clones containing the inserted PCR product were selected on the basis of white colony formation.

Plasmid DNA from three clones per transformation was prepared using the Wizard® Plasmid Mini kit (Promega Corp.) as per manufacturer's instructions. To confirm the vector contained the appropriate insert, 2 µL of a 1/200 dilution of purified plasmid DNA was amplified using blr1-F/blr1-R primers as described in 3.2.3.1. PCR products were size fractionated by 1% agarose gel electrophoresis (Appendix 7.2.5) alongside the original PCR product used in the ligation.

3.2.4.3 DNA Sequencing

One clone per transformation was sequenced at the Canterbury DNA Sequencing Facility (Canterbury University). Sequencing was performed using primers which bind to the T7 and SP6 promoter sequences flanking the multi-cloning site in pGEM®-T (Promega Corp.) (Appendix 7.5.1). To confirm identity, the sequences were aligned to the *T. atroviride* and *T. reesei blr-1* sequences using DNAman™ (Version 4.0, Lynnon Biosoft Corp., Quebec, Canada).

3.2.5 Isolation of *frq*

An annotated putative *frq* orthologue was identified in the *T. reesei* genome (scaffold 9 947100-952000 [http://genome.jgi-psf.org/Trire2/Trire2.home.html]) by conducting a Blastn search with the *N. crassa frq* sequence (ascn#: U17073). To confirm the identity of the search result, the putative *T. reesei frq* sequence was translated using Wise2 (http://www.ebi.ac.uk/Wise2) and the amino acid sequence aligned to all available *frq* sequences. Primers were designed to regions of 99% identity between the *T. reesei* and *Hypocrea spinulosa* (ascn#: U25850) (identified from a GenBank Blastp search) nucleotide sequences to amplify the *frq* gene from *T. atroviride* LU298, *T. hamatum* LU592 and *T. harzianum* LU675. Using Frq-1F (5'-TCAAGGAAGARCTGAAGCGC-3') and Frq-1R (5'-GCCATGTTGCAGAGGAGATT-3') primers, a 1 kb region, approximately 520 bp from the translational start site was amplified, cloned and sequenced in both directions from *T. hamatum* and *T. harzianum* as described in 3.2.4 except an annealing temperature of 55°C was used. To confirm identity, the sequences were aligned to the *T. reesei*, *H. spinulosa* and *N. crassa frq* sequences using DNAman™ (Version 4.0, Lynnon Biosoft, Quebec, Canada). Based on this alignment, degenerate primer Frq-2F (5'-AARAARMGNGARYTNGARGC-3') was designed to regions of 98% identity in the nucleotide sequences and used with Frq-1R to amplify *frq* from *T. atroviride*. Using these primers an 889 bp region of the *frq* gene was amplified from *T. atroviride*, cloned and sequenced. Reaction components were as described in 3.2.4.1. Amplification consisted of an initial denaturation of 5 min at 94°C, followed by 60 cycles of 30 s at 94°C, 30 s at 60°C (decreasing by 0.7°C every cycle until cycle 30 then 40°C for the remainder of cycles), and 30 s at 72°C, followed by a final extension of 10 min at 72°C. Resulting PCR products were size fractionated by 1% gel electrophoresis as described in Appendix 7.2.5. and cloned and sequenced as described in 3.2.4.2 and 3.2.4.3.

3.2.6 Isolation of *gpd*

Using the published sequence of *T. atroviride gpd* (ascn#: Y12542), primers gpd-F (5'-CGCTGCCTACATGCTCAAG-3') and gpd-R (5'-GTCAAGTCGATGACGGAGAC-3') were designed to amplify a 611 bp region located 142 bp 3' to the translational start site of the *gpd* gene from LU298. Using these primers a region of *gpd* was amplified from LU298, cloned and sequenced, as described in 3.2.4. To confirm identity, the sequence was aligned to the published *gpd* sequence using DNAman™ (Version 4.0, Lynnon Biosoft Corp., Quebec, Canada).

3.2.7 Free-running Rhythm Assay

Rhythmic gene expression was investigated in *T. atroviride* using a Free Running Rhythm (FRR) assay essentially as described for *Aspergillus nidulans*, except the experiment was extended for a longer duration (Greene *et al*., 2003). Conidial suspensions of IMI206040 (wild-type) and IMI206040 Δ*blr-2* were inoculated to deep Petri dishes at 10^5 conidia per 25 mL PDB and incubated for 72 h at 25°C in the dark. The broth was drained and the mycelial mass separated into even portions approximately 5 mm in diameter and transferred to 25 mL MM with 0.2% D-Asparagine, 0.1% NH_4NO_3 and 0.2% glucose in 100 mL conical flasks. All flasks were incubated shaking at 23°C for 24 h in constant light and then at 4 h intervals individual flasks were protected from the light. This continued over a 72 h period and 12 h after the last flask was placed into darkness all samples were snap frozen in liquid nitrogen as described in Appendix 7.2.4 and stored at -80°C. The entire experiment was repeated and sampled for an additional 48 h (120 h period in total).

3.2.8 Northern Analysis of *gpd* and *frq* Expression

3.2.8.1 RNA Extraction

Total RNA was extracted from frozen mycelium using the QIAGEN RNeasy® Plant mini Kit (QIAGEN Pty. Ltd., Clifton Hill, Vic., Australia) as per manufacturer's instructions for fungi. Each sample was ground to a fine powder in a mortar and pestle with liquid nitrogen and 100 mg used as starting material in the RNA extraction kit. All samples were stored for up to 1 week at -20°C and indefinitely at -80°C. RNA concentrations were determined spectrophotometrically using a NanaDrop® ND-1000 (NanoDrop Technologies Inc., Montchanin, DE, USA) and where the concentration was below 1500 ng per µL samples were reprecipitated and dissolved in a lesser volume as described by Sambrook *et al.* (1989).

3.2.8.2 Preparation of Northern blot

RNA was size fractionated in a MOPS-agarose-formaldehyde gel and transferred to a nylon membrane using standard laboratory techniques (Sambrook *et al*., 1989). A 1% agarose gel was prepared by dissolving 2 g Agarose in 20 mL 10 X MOPS buffer (Appendix 7.2.7) and 169 mL H_2O, cooling it to 50°C and adding 11 mL formaldehyde before pouring into a 20 x 8 cm gel casting tray containing a well-forming comb and allowed to set for ~45 min. The comb was removed and the casting tray containing the solidified gel transferred to an electrophoresis tank (E-C Apparatus Corp., New York, NY, USA) containing 1 X MOPS buffer. A 10 µL aliquot of each sample containing approximately 15 µg total RNA was mixed with 28 µL RNA sample buffer (Appendix 7.2.7) and heated to 65°C for 15 min then placed on ice for 5 min. RNA loading dye (4 µL) and 2 µL 1 mg/mL ethidium bromide were added to the denatured RNA and samples were loaded to the prepared gel, which had been pre-run for 5 min. The RNA was size fractionated by electrophoresis at a constant voltage (12.5 V/cm) for 1 h. The gel was then photographed using a Versadoc Imaging System Model 3000 (Bio-Rad Laboratories Inc., Hercules CA, USA and washed 3 X 10 min in 10 X SSX.

3.2.8.3 Capillary Transfer

A gel casting tray was placed upside down in a large pyrex dish and transfer buffer (20X SSC) added till just beneath the tray. A wick made from 3 pieces of 3MM paper (Whatman) soaked in transfer buffer was placed on top of the tray. The agarose gel was placed directly on to the wick and parafilm arranged 2-3 mm under the edges of gel. A section of Hybond-N+ nylon membrane (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) was cut to size and carefully placed on the gel avoiding airbubbles and this was covered with 3 X 3MM paper cut to size and soaked in transfer buffer. A 5 cm stack of paper towels was placed on 3MM paper, followed by a glass plate and a weight of 750 g. Capillary transfer was allowed to proceed overnight and in the morning the transfer apparatus was disassembled, the position of the wells marked on the blot with a chinograph pencil and the membrane was baked for 2 h at 80°C to fix the nucleic acid.

3.2.8.4 Labelling of *gpd* and *frq* DNA probes

Plasmid DNA containing a portion of *gpd* or *frq*, which was cloned from *T. atroviride* LU298 as described in Sections 3.2.6 and 3.2.5, respectively, were used to generate probes for the detection of mRNA on the Northern blot. The clones were used as

template in the DIG PCR Labelling System (Roche Diagnostics GmbH.) with an annealing temperature of 60°C, according to manufacturer's instructions, to generate labelled probes for chemiluminescent detection. Primers gpd-1F/gpd-1R were used to amplify a 611 bp *gpd* probe, primers FrqAtr-1F (5'-CAAGCTTAGGAGATTCACCG-3')/Frq-1R were used to amplify a 860 bp labelled *frq* probe for detection and primers PacC-1F/PacC-1R were used to amplify a 1073 bp *PacC* probe.

3.2.8.5 Chemiluminescent Detection

Chemiluminescent detection of DNA was performed using the DIG Detection system (Roche Diagnostics GmbH) according to manufacturer's instructions. For each reaction, 250 ng of DIG-labelled DNA was hybridised in tubes (Thermo Hybaid, Ashford, Middlesex, UK) overnight to the immobilised DNA in 20 mL DIG Easy Hyb. Blots encased in mylar sheets were placed in an X-ray film cassette and a sheet of Kodak BioMax XAR Film (Kodak [Australia] Pty., Ltd.) placed on top. The cassette was closed and the film exposed for 2-60 min. Films were developed manually using the Kodak GBX system (Kodak [Australia] Pty., Ltd.).

3.3 Results

3.3.1 Analysis of Rhythmic Conidial Production in *T. harzianum*

To investigate whether rhythmic conidiation occurs in *T. harzianum*, a filter paper assay was conducted using PDYC on large filter papers and Petri dishes and the light dose was given at 48 h instead of 36 h. On the control plates, mature conidiation was observed on and around the mycelial plug and in addition, two very diffuse rings of conidia were also discernible (Figure 3.2). The inner ring was green in colour, whereas in the outer ring, conidia were immature. On the light treatment plates, two conidial rings were clearly observed in addition to conidiation at the centre of the plate. The inner ring corresponded to the colony margin at the time of light exposure and in contrast to the controls, this ring was thin, well defined and intense. The outer ring represented the margin of the following day and was more mature and denser than the outer ring observed on the controls. The yellow pigment was clearly observed on all filters.

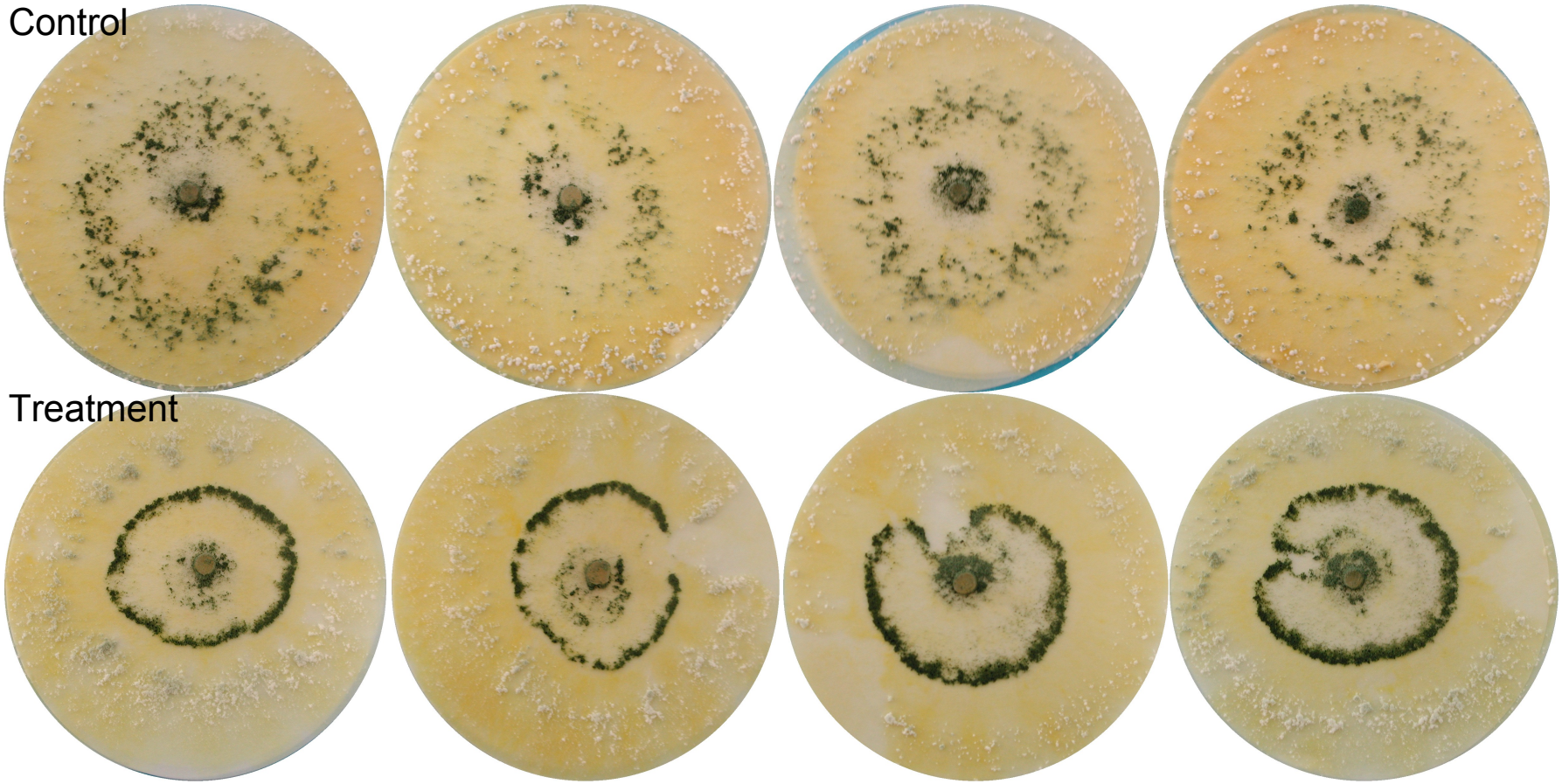


Figure 3.2. Rhythmic conidial production in *T. harzianum* on PDYC-soaked filter paper. Control = plates were grown for 6 d (144 h) in total darkness. Treatment = plates were grown for 48 h in total darkness, then photoinduced and grown a further 96 h in total darkness.

3.3.2 Analysis of Circadian Clock Gene Orthologues from *Trichoderma* spp.

3.3.2.1 *blr-1*

Primers designed to regions of homology between *T. atroviride* and *T. reesei blr-1* were successfully used to isolate a 759 bp region of the orthologous gene from *T. atroviride* LU298, *T. hamatum* LU592 and *T. harzianum* LU675. Using the primers blr1-F and blr1-R, a single band of approximately 750 bp was amplified and sequenced revealing a near perfect alignment between the reported sequence (ascn#: AY628431) and that of the *T. atroviride* presented in this chapter and a high degree of similarity between *T. atroviride blr-1* and the *T. reesei*, *T. hamatum* and *T. harzianum* sequences (Figure 3.3). All *Trichoderma blr-1* sequences isolated in this study are presented in Appendix 7.4.1.

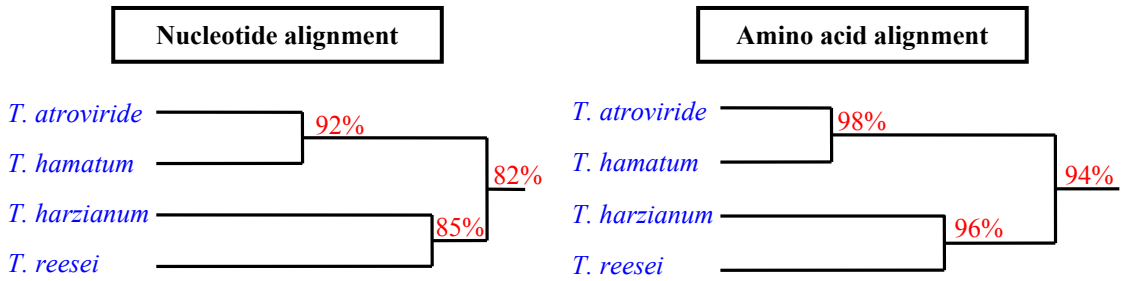


Figure 3.3. Homology trees based on sequence alignments of *blr-1* from *T. atroviride* LU298, *T. hamatum* LU592, *T. harzianum* LU675 and the *T. reesei* genome (ascn#: AY823264). Homology trees were constructed using DNAman™ (Lynnon Biosoft Corp.).

The published BLR-1 sequences from *T. atroviride* and *T. reesei* were aligned to WC-1 from *N. crassa* and analysed for the presence of protein features that have been associated with circadian clock function in *N. crassa*. The polyglutamine tracts at the N- and C-termini of WC-1 (Toyota *et al.*, 2002) were absent in both *T. atroviride* and *T. reesei*, however similar to that reported for *T. atroviride* (Casas-Flores *et al.*, 2004) the N-terminus of *T. reesei* was rich in glutamine. Interestingly, amino acid conservation between the *Trichoderma* BLR-1 and *N. crassa* WC-1 was significantly lower at the N-terminus compared with the remainder of the protein. The *Trichoderma* sequences shared 50% identity across the first 300 amino acids, compared with 88% across the remainder of the protein. Similarly, *T. atroviride* BLR-1 shared only 12.5% identity with *N. crassa* WC-1 across the first 300 amino acids, whereas they shared 61% identity

across the remaining sequence. In contrast, four of the five phosphorylation sites that are associated with circadian clock function in *N. crassa* (He *et al.*, 2005a) were conserved in both *T. atroviride* and *T. reesei* (Figure 3.4).

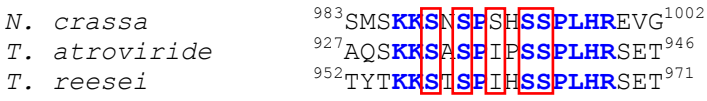


Figure 3.4. Alignment of the phosphorylation domain in WC-1 from *N. crassa* (ascn#: X94300) and BLR-1 from *T. atroviride* (ascn#: AY628431) and *T. reesei* (ascn#: AY823264). Amino acids in blue are idenitical between all three isolates and phosphorylation sites in *N. crassa* are boxed in red.

3.3.2.2 *frq*

Using a combination of genome mining and targeted PCR, putative orthologues of the *frq* gene were identified from *T. reesei*, *T. atroviride*, *T. hamatum* and *T. harzianum*. Blast analysis of the *T. reesei* genome identified an annotated putative *frq* sequence with approximately 48% amino acid identity to *N. crassa* FRQ and 72% identity to *H. spinulosa* FRQ. On the basis of the alignment between *H. spinulosa* and *T. reesei frq*, primers were designed and successfully used to isolate the orthologous gene from *T. hamatum* and *T. harzianum*. Using the primers Frq-1F and Frq-1R, a single band of 981 bp and 1024 bp were amplified and sequenced from *T. hamatum* and *T. harzianum*, respectively, revealing a high degree of similarity to *frq* from *T. reesei* and other filamentous fungi. Amplification of *frq* from *T. atroviride* with Frq-1F and Frq-1R was unsuccessful; therefore new primers were designed on the basis of alignment between *H. spinulosa*, *T. reesei*, *T. hamatum* and *T. harzianum frq*. Primers Frq-2F and Frq-1R were successfully used to amplify an 889 bp region of the *frq* gene from *T. atroviride*. Alignment of the *Trichoderma* spp. *frq* sequences to those from other filamentous fungi revealed a high degree of conservation at both the nucleotide and amino acid level (Figure 3.5), however, this was considerably less than that observed between *blr-1* orthologues. All *Trichoderma frq* sequences isolated in this study are presented in Appendix 7.4.2.

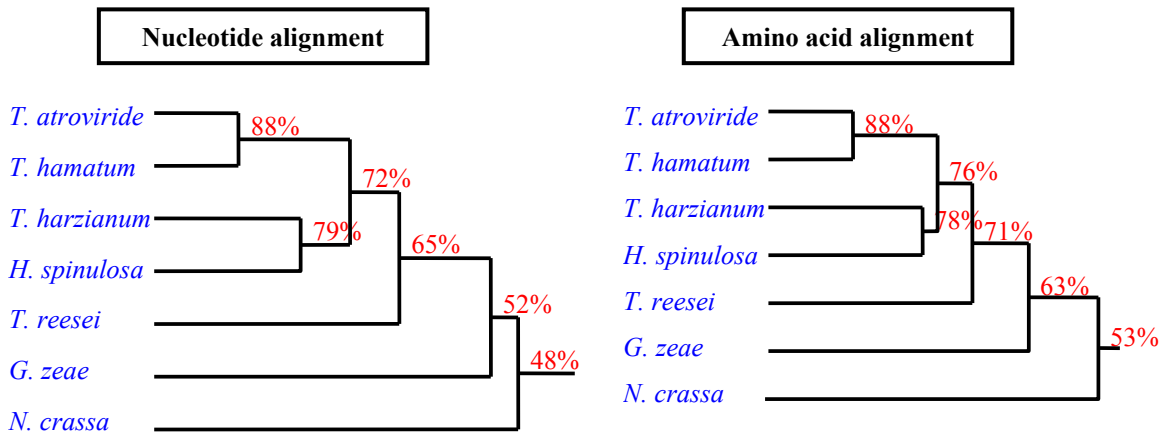


Figure 3.5. Homology trees based on sequence alignments of *frq* from *T. atroviride* LU298, *T. hamatum* LU592, *T. harzianum* LU675, *H. spinulosa* (ascn#: CSU25850), the *T. reesei* genome (this study), *G. zeae* (ascn#: AACM01000240) and *N. crassa* (ascn#: BX908789). Homology trees were constructed using DNAman™ (Lynnon Biosoft Corp.).

The promoter sequence of the putative *frq* gene from *T. reesei* was analysed for the presence of light-responsive elements (LREs) (GATNC----CGATN) (He & Liu, 2005b). In *N. crassa*, light input to the circadian system is mediated by binding of the WC-1/WC-2 complex to the *frq* promoter at the consensus LREs (Froehlich *et al.*, 2002). No consensus LRE elements were identified in the *T. reesei frq* promoter.

3.3.2.3 *gpd*

Primers designed from the published *gpd* sequence of *T. atroviride* IMI206040 (ascn#: Y12542) were successfully used to isolate a 611 bp region of the *gpd* gene from *T. atroviride* LU298. Using primers gpd-F and gpd-R a single band of approximately 600 bp was amplified and sequenced revealing 100% identity between the reported *T. atroviride* sequence and that of LU298 (data not shown).

The 5' flanking sequence of *gpd* from *T. atroviride* was not available, however, approximately 1.4 kb upstream of the translational start site have been published for *T. reesei* (ascn#: EF043568). Sequence analysis of the *T. reesei* regulatory region revealed the presence of one consensus LRE motif at -789, relative to the transcriptional start: GATAC$(N)_{22}$CGATT(-789). The *N. crassa gpd* upstream sequence (ascn#: AL670003) was also analysed for LREs and, as in *T. reesei*, a single consensus motif was identified: GATGC$(N)_5$CGATG(-932).

3.3.3 Analysis of Rhythmic Gene Expression in *T. atroviride*

Using a free-running rhythm (FRR) assay, *gpd* expression was analysed in *T. atroviride* and a *Δblr-2* mutant derivative. Similar to that reported for *N. crassa* and *A. nidulans*, expression of *gpd* was rhythmic in the wild-type (Figure 3.6). *gpd* was highly expressed from 16-48 h and from 72-80 h following transfer to the dark and was clearly down-regulated for 24 h in between. Expression also appeared down-regulated at both 12 h and 84 h, however on the basis of a single lane, it was not possible to determine if this represented a trend. In contrast, the rhythm observed in the wild-type was absent in the Δ*blr-2* mutant, though expression was still high. No clear trend in expression was observed.

The experiment was repeated and continued for an extra 48 h to determine whether the rhythm observed in the wild-type continued. Unfortunately, however, the RNA quantity and quality was insufficient for RNA analysis.

The *frq* gene has been shown to exhibit a rhythm in *N. crassa* and this rhythm was significantly altered in a Δ*wc-2* mutant (Collett *et al.*, 2002). The FRR assay northern blots were probed for *frq* expression, however no signal was detected at any time point in *T. atroviride* and the *Δblr-2* mutant.

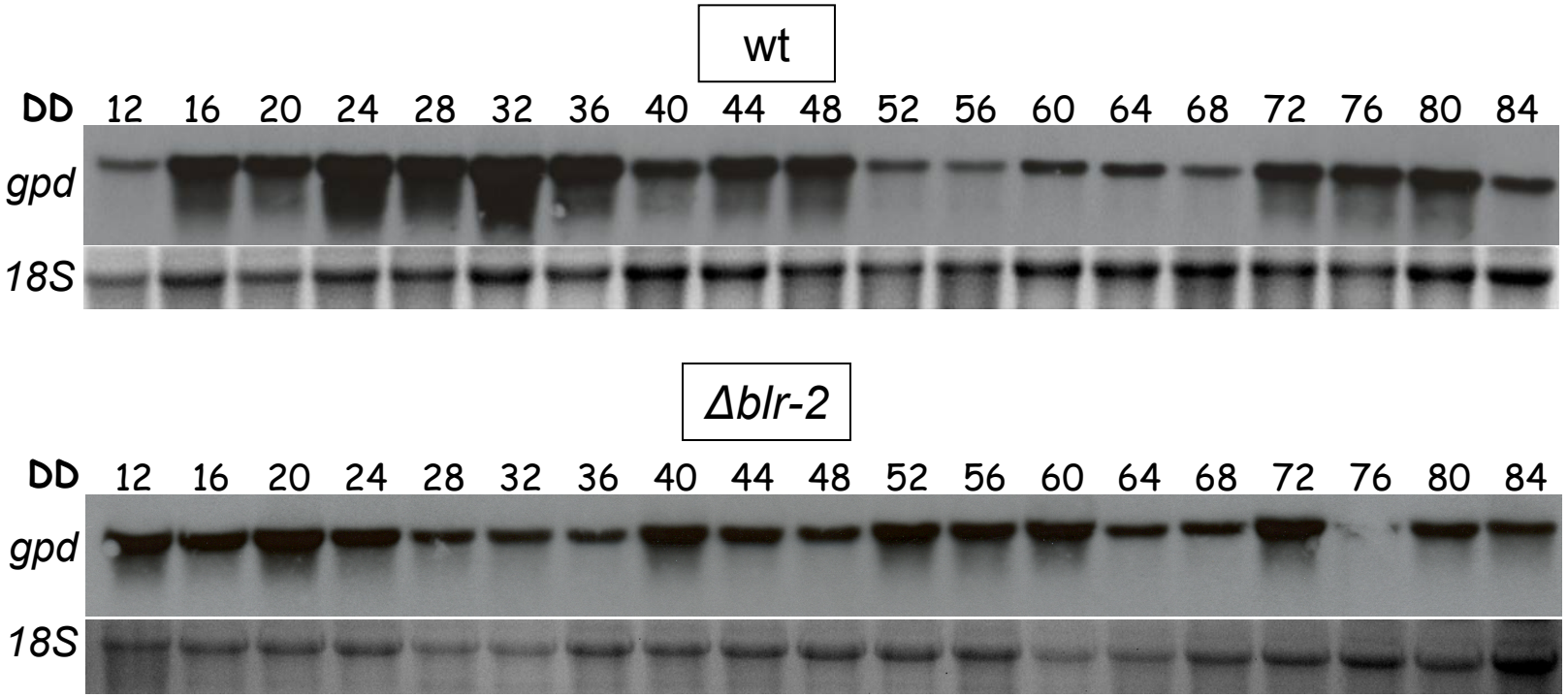


Figure 3.6. Expression of *T. atroviride gpd* in a free-running rhythm assay. Total RNA was extracted from *T. atroviride* IMI206040 wild-type (wt) and *Δblr-2* derivative cultures grown in 24 h light, then transferred to constant darkness (DD) for the hours indicated on the figure. 15 µg total RNA was loaded into each lane and the resulting blots were probed with a PCR product derived from the *T. atroviride gpd* gene to identify the *gpd* mRNA transcript. Ethidium bromide-stained 18S rRNA is shown as a loading control.

3.4 Discussion

3.4.1 Conidiation is Rhythmic in *T. harzianum*

On large PDYC-soaked filter papers, *T. harzianum* produced a ring of conidia from hyphae which formed approximately 24 h after the light exposure, which strongly suggested light-induction of rhythmic conidiation, however rhythmic conidiation also occurred in the absence of light. Though not clearly defined, two diffuse ring-like arrangements of conidia were clearly observed on the controls and similar to the light treatment plates the first ring appeared after 48 h and the second ring after at least 72 h growth. These results clearly demonstrate that conidiation can be rhythmic, and possibly circadian, in *T. harzianum* and although light enhanced the conidiation rhythm, contrary to our hypothesis, it did not induce it. To the best of our knowledge, this is the first report of rhythmic, possibly circadian, conidiation in *T. harzianum*.

Rhythmic conidiation in the absence of light strongly suggests the presence of an endogenous rhythm associated with conidiation. Deitzer *et al.* (1988) observed an endogenous rhythm in light sensitivity in *T. atroviride*, as measured by photo-induced conidiation, but no rhythmic conidiation was observed in the dark and conidiation in rings was light-inducible only. In Chapter 2, conidiation appeared to cluster into two rings in *T. atroviride* LU298 on PDA buffered to pH 2.8, when light was given to cultures of sufficient age, which suggests a possible endogenous light sensitivity in these cultures. Together these results suggest differences in conidiation-associated endogenous rhythms between *T. harzianum* and *T. atroviride*, however rhythmic conidiation was not observed in *T. harzianum* under all conditions in this study. Indeed on broth-soaked filter paper, no rings of conidia were observed in the dark when PDB was the medium, but were clearly observed when the broth was amended with yeast extract and casein hydrolysate. It is possible that rhythmic dark conidiation may also occur in other *Trichoderma* spp., but the appropriate conditions under which this may be observed have yet to be identified.

It would appear that the observed conidiation rhythm in *T. harzianum* is endogenous, however it also possible that an aspect of the mycelial plug may have induced rhythmic conidiation. In Chapter 2, a ring of conidia on the control plates was observed in *T. harzianum* on PDA buffered to pH 2.8 and 3.2 when mycelial plugs were used as inoculum, but was absent when plates were inoculated with a conidial suspension (2.11.5). Exposure of the mycelial plug to light was ruled out as a possible cause of dark

conidiation on PDYC-soaked filter paper by this isolate (2.15.1.1). It is possible that another factor associated with the plug, such as age of the inoculum, may have stimulated the rhythm, or the use of mycelial plugs reveals rhythmic conidiation under certain conditions. In *Fusarium oxysporum*, it was demonstrated that the use of mycelial plugs greatly increased variability in morphology compared with conidial inoculum and that the age of the colony from which the mycelial plugs were taken also affected variability (Awuah & Lorbeer, 1989). The choice of inoculum affected conidiation in the dark in *T. atroviride* on PDA buffered to pH 2.8, where mycelial inoculum appeared to negatively influence conidiation in the dark over time (2.11.3). In contrast to *T. atroviride*, the use of mycelial plugs appeared to promote rather than inhibit dark conidiation in *T. harzianum*, which suggests that mycelial plugs differentially affect conidiation between these two isolates.

3.4.2 *gpd* Expression is Rhythmic in *T. atroviride*

Following transfer from constant light to constant dark, *T. atroviride gpd* expression, in accordance with the proposed hypothesis, exhibited a free-running rhythm. The length of the observed free-running period (FRP) was between 48 and 56 h, however high *gpd* expression was sustained for 36 h in the first peak and at least 12 h in the second, which suggests that the period reduced over time. A second experiment with extended sampling times will be required to definitively establish the period length. Nevertheless, the *gpd* FRP observed in this study, was considerably longer than in both *N. crassa* (22 h) and *A. nidulans* (28-32 h) (Shinohara *et al*., 1998; Greene *et al.*, 2003). The two day free-running rhythm in *T. atroviride* was surprising, as rhythmic *gpd* expression is, in other species, considered circadian and circadian rhythms are typically closer to 24 h (Greene *et al*., 2003). Indeed, the longest reported free-running circadian rhythm was, until now, that of sclerotial development in *A. flavus* (33 h) (Greene *et al.*, 2003). These authors discussed how their findings question the assumption that circadian rhythms have FRPs close to 24 h. The results from the *gpd* FRR experiment clearly demonstrate that although rhythmic expression of *gpd* is conserved in *T. atroviride*, *N. crassa* and *A. nidulans*, the length of the FRP is species-specific and not confined to approximately 24 h.

In both *A. nidulans* and *N. crassa*, the *gpd* rhythm is entrainable by light (Shinohara *et al.*, 1998; Greene *et al.*, 2003), which is a second defining feature of circadian rhythms. It was not determined if *gpd* expression is entrainable in *T. atroviride*, however

conidiation is entrainable in all *Trichoderma* species and is a characteristic feature of this genus. In this study, conidiation was demonstrated to exhibit a free-running rhythm in *T. harzianum* using a modified PDYC-soaked filter assay. In the same assay, conidiation was arrhythmic in *T. atroviride* LU298 (data not shown). Similarly, *gpd* is rhythmically expressed in *A. nidulans*, however conidiation is light entrainable but arrhythmic, yet in *A. flavus*, sclerotial production is clearly rhythmic (Greene *et al.*, 2003). These authors suggest that while there are similarities in the circadian clocks of these two aspergilli, development is not under the control of an endogenous clock in *A. nidulans*. Both rhythmic conidiation in *T. harzianum* (this study) and sclerotial development in *A. flavus* (Greene *et al.*, 2003) are nutritionally dependent. It is possible, therefore, that absence of developmental rhythms in *T. atroviride* and *A. nidulans* is due to a lack of knowledge of the appropriate environmental conditions.

3.4.3 Rhythmic *gpd* Expression is *blr-2* Dependent

The *gpd* rhythm observed in the free-running rhythm (FRR) assay was absent in a *Δblr-2* mutant derivative, which strongly suggests that the wild-type *gpd* rhythm requires an active BLR-2. In *T. atroviride Δblr-2*, *gpd* expression was variable, however no pattern was discernible. Repetition of this experiment will resolve whether the *gpd* rhythm is random or altered in a *Δblr-2* background. To the best of our knowledge, the expression patterns presented in this chapter represent the first study on the *gpd* free-running rhythm in a *blr-2* or *wc-2* defective background.

In *A. nidulans* it was reported that in preliminary studies, *gpd* was rhythmic in a *ΔireA* (*wc-1* orthologue) background, however no data was shown (Greene *et al.*, 2003). If the endogenous clock controlling *gpd* expression in *T. atroviride* is similarly regulated to the *A. nidulans* clock, then this would suggest that BLR-2 (IreB) participates in the rhythm independently of BLR-1 (IreA). It is more likely that in *A. nidulans*, the *gpd* rhythm is not IreA/IreB associated and in *T. atroviride*, the wild-type *gpd* free-running rhythm requires an active BLR-1/BLR-2 complex. Indeed the involvement of BLR-1/BLR-2 is supported by the presence of conserved circadian-associated domains in the *T. atroviride* and *T. reesei* protein sequences (3.3.2.1).

In *N. crassa*, the FREQUENCY protein is an integral part of the circadian clock. Light resets the circadian clock through the binding of the WC complex to the FRQ promoter (Froehlich *et al.*, 2002). *Frq* expression is light-induced and exhibits a free-running

rhythm, and this is WC-2 dependent (Collett *et al.*, 2002). In this study, putative gene orthologues, with high similarity to *N. crassa frq*, were identified in three *Trichoderma* species and from the *T. reesei* genome. Attempts were made to investigate *frq* expression in the free-running rhythm assay, however no signal was detected on either the wild-type or *Δblr-2* northern blots. Indeed, sequence analysis of *T. reesei frq* and *gpd* revealed no conserved circadian or light-responsive elements (LREs) in the *frq* promoter, and one conserved LRE in the *gpd* promoter, which is in agreement with our northern analysis of these two genes. The lack of *frq* expression in *T. atroviride* and the absence of conserved LREs in the *T. reesei frq* promoter suggests that in *T. atroviride*, FRQ is not involved in the circadian clock. This was also asserted for *A. nidulans* in which no *frq* orthologue was able to be idenitified (Greene *et al*., 2003). These authors discuss how *frq* orthologues have not been identified to date in the Plectomycetous subgroup of Acomycotina, whereas multiple orthologues have been identified in Pyrenomycetous species, including *N. crassa*, and suggest that the FRQ-based oscillator is not conserved amongst the Ascomycotina subgroups. On the basis of the literature and results from this study, it is tempting to speculate that while circadian clocks are conserved in all fungi, involvement of a WC complex is conserved within subgroupings of the Ascomycotina and the FRQ-base oscillator is conserved in *N. crassa* only.

Chapter Four
Molecular Analysis of *Trichoderma* spp. *rcoT* (*tup1*)

4.1 Introduction

The Tup1 protein forms a complex with Ssn6 (Cyc8), which acts as a global repressor of many regulatory pathways within the yeast cell. The Tup1-Ssn6 complex is composed of 3-4 molecules of Tup1 and 1 molecule of Ssn6 (Redd *et al.*, 1997). Tup1 is classed as a WD-40 repeat protein. This ancient family of regulatory proteins has a diversity of functions. They are found in all Eukaryotic cells, but are absent in Prokaryotes (Neer *et al.*, 1994). WD-40 repeat proteins contain 4-8 conserved WD-40 domains, which are characterised by a core sequence beginning with Gly-His (GH) and ending in Trp-Asp (WD): $\{X_{6\text{-}94} - [GH - X_{23\text{-}41} - WD]\}$ (Neer *et al.*, 1994). Members of this family also include the G-proteins, which are involved in signal transduction of multiple stimuli. The 7 WD-40 repeats of Tup1 form a propeller-like structure, which has been shown to be important for interaction with other proteins. The N-terminus of Tup1 contains a coiled coil domain, which forms a helical structure important for Tup1 tetramerisation and interactions with Ssn6. Between the coiled coil domain and the WD-repeats lies a glutamine- and alanine-rich region which participates in histone binding and gene repression. Ssn6 is a tetratricho peptide repeat (TPR) protein, containing a TPR motif with 10 tandem repeats (Schultz *et al.*, 1990). TPR motifs result in the formation of a superhelical structure, which mediates protein-protein interactions (D'Andrea & Regan, 2003). In Ssn6, the TPR motifs mediate interactions with other transcriptional repressors (Tzamarias & Struhl, 1995) and aid in the formation of the Tup1-Ssn6 complex (Jabet *et al.*, 2000).

Rather than binding directly to the promoters of Tup1-Ssn6 regulated genes, the Tup1-Ssn6 complex is recruited to the promoter by pathway–specific, negative regulators. The TPR motifs in Ssn6 are essential for this recruitment as they bind to the negative transcription factors (Tzamarias & Struhl, 1995). Repression is achieved either by altering chromatin structure through Tup1 interacting with histones and histone deacetylases or by Tup1 interacting with the RNA Polymerase II mediator complex (Zhang & Reese, 2004). Many pathways are negatively regulated in this fashion by Tup1-Ssn6 in the yeast, including mating-type specificity, DNA damage response, dimorphism and glucose repression (Malavé & Dent, 2006, and references therein).

Indeed, approximately 3% of all genes in *S. cereviseae* are repressed by the Tup1-Ssn6 complex (Smith & Johnson, 2000). Orthologues of Tup1 and Ssn6 have been identified in yeasts, filamentous fungi and worms and although no conserved orthologues have been identified in higher Eukaryotes, corepressor proteins with similar domain structures and function have been identified in flies and vertebrates (Malavé & Dent, 2006).

Tup1 was first identified in filamentous fungi as a negative regulator of conidiation-specific genes. Early studies on reproduction in *N. crassa* identified multiple genes which are expressed preferentially during conidiation (*con* genes) (Berlin & Yanofsky, 1985). In a study designed to identify mutants in which *con-10* was aberrantly expressed, *rco-1* (*tup1*) was isolated as a negative regulator of *con-10* expression (Yamashiro *et al.*, 1996; Madi *et al.*, 1994). In the *rco-1* mutant background, *con-10* expression, which is normally exclusive to conidiophore and conidia, was observed in hyphal cells (Yamashiro *et al.*, 1996). In response to carbon or nitrogen starvation, or heat shock, *con-10* transcript levels were further increased in *rco-1* mutants (Lee & Ebbole, 1998). These authors suggest that Rco-1 mediates *con-10* expression in response to both developmental and environmental signals.

In addition to affecting *con-10* expression, loss of *rco-1* had a dramatic effect on culture morphology and sporulation. The growth rate of all mutants was nearly one fifth that of the wild-type and the hyphal coiling pattern observed in the wild-type was reversed to an anticlockwise direction (Yamashiro *et al.*, 1996). Mutants were either aconidial, or produced conidiophores from which the conidia did not detach, and were female sterile. Whether the effect on sporulation is related to aberrant *con-10* or other targets of Rco-1 is not known, however *con-10* is expressed during both asexual and sexual reproduction (Springer & Yanofsky, 1992), which clearly demonstrated that Rco-1 regulates genes involved in both pathways (Yamashiro *et al.*, 1996).

Asexual and sexual reproduction is also regulated by the Tup1 orthologue in *A. nidulans*, RcoA. Conidiation in a Δ*rcoA* strain was poor compared to the wild-type and mutants were self sterile (Hicks *et al.*, 2001; Richard Todd, pers. comm., unpublished). Altered expression of a conidiation gene was also observed. In *A. nidulans*, BrlA is a transcription factor which regulates conidiophore development (Adams *et al.*, 1988). In Δ*rcoA* mutants, *brlA* expression is delayed by 84 h. In addition, the *A. nidulans* Δ*rcoA*

mutants were reduced in growth compared to the wild-type (one tenth) and the colony margins were irregular indicating that like *N. crassa* Rco-1, RcoA plays a major role in growth and development (Hicks *et al.*, 2001).

Interestingly, RcoA is essential for sterimatocystin (ST) production, which suggests that RcoA plays a regulatory role in secondary metabolism. ST production and expression of two key genes were absent in Δ*rcoA* mutants, which suggested a positive regulatory role for RcoA (Hicks *et al*., 2001). Transcriptional activation of some target genes by Tup1 has been observed in *S. cerevisiae* (Conlan *et al.*, 1999), however Hicks *et al.* (2001) also suggested that RcoA may interact with other pathways which affect ST production.

Loss of function of the *tup1* orthologue, *tupA*, resulted in severe growth defects in *Penicillium marneffei*, however in contrast to both *N. crassa* and *A. nidulans*, asexual reproduction was promoted (Todd *et al.*, 2003). Conidiation was initiated after 3 d in a Δ*tupA* mutant compared with 7 d in the wild-type. Expression of the conidiation transcription factor, *brlA*, was also earlier than in the wild-type *P. marneffei*. No sexual stage has been observed in *P. marneffei*, however *tupA* can fully complement the sexual dysfunction in an *A. nidulans* Δ*rcoA* mutant (Todd *et al.*, 2003). In *N. crassa*, *rco-1* is also necessary for sexual reproduction (Yamashiro *et al.*, 1996), which suggests that the sexual function of *tup1* orthologues is conserved in the filamentous fungi.

At 37°C*, P. marneffei* switches from filamentous growth to a reversible yeast phase (Garrison & Boyd, 1973) and in *P. marneffei* Δ*tupA* mutants, filamentation was reduced and aberrant yeast formation occurred at 25°C (Todd *et al.*, 2003). This differed from *C. albicans*, in which filamentous growth was constitutive in a Δ*tup1* background (Braun & Johnson, 1997). Yeast colony growth rate was also severely reduced in the *P. marneffei* Δ*tupA* mutants, compared with the wild-type and microscopy revealed irregularities in the yeast cell walls, which were associated with chitin deposits.

It is not known if the Tup1 orthologues (Rco-1, RcoA & TupA) are involved in chromatin remodelling, however many similarities exist between the sequence of this protein in yeasts and filamentous fungi (Yamashiro, *et al.*, Hicks *et al.*, 2001 & Todd *et al.*, 2003). All proteins contain 7 WD-40 domains and each domain is more similar to the equivalent domain in other species than to domains within the same sequence, which is a characteristic feature of WD-40 repeat protein families (Neer *et al.*, 1994). A

conserved coiled coil domain is present at the N-terminus of all proteins, which in yeasts is important for formation of the Tup1-Ssn6 structure. The region between the coiled coil domain and the WD-40 repeats is rich in proline, which has been suggested to act as a functional equivalent to the glutamine- and alanine-rich histone binding region in Tup1 (Yamashiro *et al.*, 1996). A putative *Ssn6* orthologue, *rcm-1*, has been idenitifed in *N. crassa* (Genbank accession number: AY576485 [http://www.ncbi.nlm.gov/Genbank]), however no studies have been published in association with the sequence. The high level of sequence similarity between the Tup1 proteins strongly suggests they are indeed orthologous.

SSN6 orthologues have recently been identified through genome annotation, however there are no published studies as yet on the function of this orthologue in the ascomycetous fungi. In the basidiomycetous fungus *Ustilago maydis*, a SSN6 orthologue, Sql1, is involved in repression of cAMP-regulated genes (Loubradou *et al.*, 2001).

4.1.1 Objectives of this Study

The objectives of this study were to isolate the *rco-1* (*tup*-1) orthologue from *T. atroviride* and *T. hamatum* and use mutational analysis to investigate a role in conidiation. Based on results from Part A, a role in both light- and injury-induced conidiation will be investigated. It was hypothesised that a highly conserved copy of *rco-1* was present in *Trichoderma* (*rcoT*) and that *rcoT* will also act as key regulator of conidiation in this genus. It was also hypothesised that highly conserved orthologues of the yeast co-regulator gene *ssn6* and the *N. crassa con-10* conidiation gene would also be present in species of *Trichoderma* and that *con-10* would be expressed in hyphae from *Trichoderma* spp. *rcoT* mutants. In *N. crassa* Rco-1 has been demonstrated to be a negative regulator of conidiation-specific genes. Analysis of the Rco-1 orthologue in *Trichoderma* spp. will likely provide insight into the regulation of conidiation within these species.

4.2 Materials and Methods

4.2.1 Fungal Isolates

Two *Trichoderma* biocontrol isolates from New Zealand were obtained from the Lincoln University culture collection for use in this study. The isolates were *T. atroviride* LU298 and *T. hamatum* LU592. Isolate origins are presented in Table 2.1, section 2.2.1. All isolates had been previously identified on the basis of morphology and ITS sequencing (Kirstin McLean, pers. comm.). Isolates were maintained and stored on potato-dextrose agar (PDA) (Appendix 7.2.1.).

4.2.2 Extraction of Genomic DNA for use in PCR Amplifications

Genomic DNA was isolated from *T. atroviride* and *T. hamatum* (2.2.1) using the Puregene® Genomic DNA Purification Kit (Gentra Systems Inc.) as per manufacturer's instructions. Approximately 200 mg of frozen mycelium (Appendix 7.2.4) was placed in a sterile mortar and pestle containing liquid nitrogen. The mycelium was ground to a fine powder and 20 mg transferred to a chilled centrifuge tube containing 600 µL of cell lysis buffer, 30 µg RNaseA and 60 µg Proteinase K (Roche Diagnostics GmbH.). The mixture was immediately homogenised using a wide-bore pipette tip, and placed on ice. Samples were incubated at 55°C for 60 min, with inversion of tubes once. Tubes were cooled to room temperature and 200 µL protein precipitation buffer added. Tubes were inverted 10 times and placed on ice for 15 min. The solution was centrifuged at 20 000 x*g* for 5 min and the supernatant containing DNA transferred to a fresh tube. DNA was precipitated by addition of one volume of 100% isopropanol and incubation on ice for 5 min, then pelleted by centrifugation at 20 000 x*g* for 10 min at 4°C. The pellet was washed twice in 70% ethanol, air-dried, resuspended in 100 µL of sterile water and stored at 4° C. DNA prepared for use in inverse PCR was precipitated with two volumes of 100% ethanol rather than isopropanol, as the latter can inhibit restriction digestion (Sambrook *et al.*, 1989). DNA samples were quantified via gel electrophoresis as described in Appendix 7.2.5.

4.2.3 Isolation of *rcoT*

4.2.3.1 Degenerate Primer Design

Degenerate primers were designed to regions of 100% identity in the protein sequence of *N. crassa* Rco1, *A. nidulans* and *P. marnefeii* TupA, and *G. zeae* and *M. grisea* hypothetical proteins (ascn#: AAB37245, AAG28504, AAL99251, XP382677 and EAA46486 respectively) and predicted to amplify a 585 bp region of the *T. atroviride*

and *T. hamatum rcoT* genes. Sequences were analysed using DNAman™ (Version 4.0, Lynnon Biosoft Corp.). Two criteria were used to select appropriate regions from the aligned protein sequences for degenerate primer design. First, they must contain two or more of the amino acids Phe, Tyr, Gln, Asn, Asp, His, Cys, Lys, Glu, Met and Trp which exhibit little or no codon degeneracy. Second, the 3' nucleotide must not be degenerate. Primers Rco384pS (5'-GGNCAYGARCARGAYATHTA-3') and Rco554pAS (5'-NGGRTCCCARAAYTGNACNC-3') were designed to the selected regions (Figure 4.1).

Amino Acid	^{384} Gly His Glu Gln Asp Ile Tyr ^{390}
Rco384pS	5' ggN caY gaR caR gaY atH ta 3'
Amino Acid	^{548} Gly Val Gln Phe Trp Asp Pro ^{554}
Nucleotide (sense)	5' ggN gtN caR ttY tgg gaY ccN 3'
Rco554pAS (antisense)	3' cN caN gtY aaR acc ctR ggN 5'

Figure 4.1. *rcoT* degenerate primer design. Blue represents the selected amino acid regions. Numbering is relative to the *N. crassa* Rco1 protein sequence (ascn#: AAB37245). Degenerate primers are in red. N = a/t/g/c, Y = c/t, R = a/g, H = a/c/t.

4.2.3.2 Amplification of *rcoT*

Using the primer set Rco384pS/Rco554pAS, ~500 bp portion of the *rcoT* gene was amplified from *T. atroviride* LU298 and *T. hamatum* LU592. All PCR amplifications were performed in a Bio-Rad Icycler™ (Bio-Rad Laboratories Inc.). Each 25 µL PCR reaction contained 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM MgCl2, 200 µM each of dATP, dCTP, dGTP and dTTP (Roche Diagnostics GmbH.), 25 pmoles of each primer, 10 ng of *T. atroviride* or *T. hamatum* DNA and 1.25 U Taq DNA polymerase (Roche Diagnostics GmbH.). DNA from *N. crassa* was included as a positive control. To check for contamination, a negative control was included that contained all the above ingredients except template DNA. Single primer controls containing all the above ingredients and only one of the two primers were also included, to assess whether one primer could amplify in both directions.

Amplification consisted of an initial denaturation of 5 min at 94°C, followed by 60 cycles of 30 s at 94°C, 30 s at 60°C (decreasing by 0.7°C every cycle until cycle 30 then 40°C for the remainder of cycles), and 30 s at 72°C, followed by a final extension of 10 min at 72°C. Resulting PCR products were size fractionated by electrophoresis through a 1% agarose gel as described in Appendix 7.2.5.

4.2.3.3 TA Cloning of PCR Products

PCR products obtained as described were ligated into the TA-cloning site in pGEM®-T (Promega Corp.) (Appendix 7.5.1) as per manufacturer's instructions and transformed into *E. coli* strain INVαF' (Invitrogen Corp.) using standard techniques (Sambrook *et al.*, 1989). Typically, a 2 μL aliquot of the ligation was mixed with 40 μL of competent cells and incubated on ice for 30 min. Cells were heat shocked at 42°C for 40 s to facilitate DNA uptake, then placed on ice for 2 min. A 350 μL aliquot of SOC medium (Appendix 7.2.6) was added and transformations incubated at 37°C for 1 h to allow ampicillin resistance gene expression. A 100 μL aliquot of the transformation mix was spread to LB/Amp/X-Gal selection plates (Appendix 7.2.6) and incubated overnight at 37°C. Clones containing the inserted PCR product were selected on the basis of white colony formation.

Plasmid DNA from three clones per transformation was prepared using the Wizard® Plasmid Mini kit (Promega Corp.) as per manufacturer's instructions. To confirm the vector contained the appropriate insert, 2 μL of a 1/200 dilution of purified plasmid DNA was amplified using Rco384pS/Rco554pAS as described in 4.2.3.2 except that a standard PCR amplification protocol was used. Amplification consisted of an initial denaturation of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C, followed by a final extension of 5 min at 72°C. PCR products were size fractionated by 1% agarose gel electrophoresis (Appendix 7.2.5) alongside the original PCR product used in the ligation.

4.2.3.4 DNA Sequencing

One clone per transformation was sequenced at the Canterbury DNA Sequencing Facility (Canterbury University). Sequencing was performed using both the T7 and SP6 promoters flanking the multi-cloning site in pGEM®-T (Promega Corp.) (Appendix 7.5.1). To confirm the identity of the DNA insert, the primer sequences were removed and the sequence subjected to a Blastn search in GenBank (http://www.ncbi.nlm.gov/Genbank).

4.2.3.5 Derivation of the Full Gene Sequence using Inverse PCR

Trichoderma atroviride

The *T. atroviride rcoT* sequence derived above was analysed using DNAman (version 4.0, Lynnon Biosoft Corp.) for non-cutting 6 bp restriction enzyme recognition sequences. A 500 ng aliquot of *T. atroviride* LU298 gDNA was digested to completion using *Pst*I (Roche Diagnostics GmbH.), according to manufacturer's instructions, in a total volume of 50 µL. Digestion was confirmed by 1% agarose gel electrophoresis of a 10 µL aliquot alongside 50 ng of uncut gDNA. The restriction enzyme was removed by phenol/chloroform extraction (Appendix 7.2.4) and the digested DNA reprecipitated using 1/10 volume 3 M sodium acetate and two volumes 100% ethanol, followed by incubation on ice for 15 min. DNA was pelleted by centrifugation at 14 000 x*g* at 4°C, the pellet washed three times in 70% ethanol, air dried and re-dissolved in 100 µL sterile distilled water.

Digested DNA was circularised by ligation with 1 U T4 DNA ligase (Roche Diagnostics GmbH.), according to manufacturer's instructions, in a total volume of 250 µL. The ligase was removed by phenol/chloroform extraction (Appendix 7.2.4), the DNA reprecipitated (as described above) and re-dissolved in 50 µL sterile distilled water. All DNA was stored at -20°C.

Inverse primers Irco1R (5'-AGACGCGAACGCTCTTGTC-3') and Irco1F (5'-AAGATGTGGGAGCTTAGCTC-3') were designed to regions of 100% identity approximately 50 bp inside the known *T. atroviride* LU298 and *T. hamatum* LU592 *rcoT* DNA sequences. Each 25 µL reaction contained 1 X Expand Long Template buffer 2 (Roche Diagnostics GmbH.), 500 µM each of dATP, dCTP, dGTP and dTTP (Roche Diagnostics GmbH.), 7.5 pmoles of each primer, 50 ng of circularised restriction-digested DNA and 0.75 µL Expand Long Template Enzyme mix (3.75 U). Amplification consisted of an initial denaturation of 2 min at 94°C, followed by 35 cycles of 10 s at 94°C, 30 s at 60°C and 2 min at 68°C (increasing by 20 s every cycle after 10 cycles), followed by a final extension of 7 min at 68°C. A gDNA control (10 ng) was included to assess possible amplification of any undigested DNA. Inverse-PCR products size fractionated by 0.8% agarose gel electrophoresis as described in Appendix 7.2.5.

Amplification of the *Pst*I prepared template yielded an 8.5 kb inverse PCR product, which was purified from 1% agarose using the QIAEX II Gel Extraction Kit (QIAGEN Pty. Ltd.) according to manufacturer's instructions and the DNA reprecipitated (as described above) and re-dissolved in 7 µL sterile distilled water. The purified DNA was then A-tailed in a 10 µL reaction which consisted of 7 µL purified PCR product, 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of dATP and 5 U Taq DNA Polymerase (Roche Diagnostics GmbH.). Components were mixed on ice and then incubated at 70°C for 30 min in a Bio-Rad Icycler™ (Bio-Rad Laboratories Inc.).

The A-tailed *Pst*I inverse PCR product was ligated to pGEM®-T (Promega Corp.) and transformed into the *E. coli* strain INVαF' (Invitrogen Corp.) as described in section 4.2.3.3 Circularisation of the restriction fragments should have reconstructed the restriction site, and this was confirmed by digesting of plasmid DNA with *Pst*I according to manufacturer's instructions. Restriction patterns were examined by 0.8% agarose gel electrophoresis. Plasmid DNA from clones containing two restriction sites were sequenced using the SP6 and T7 primers as described in section 4.2.3.4 and an extended ~ 1300 bp sequence of *T. atroviride rcoT* was compiled.

The *Pst*I clone was further sequenced in both directions using the *rcoT* specific primers 298rcoIx2R (5'-CGAACACAGCAAACGACGC-3'), 298rcoIx2R (5'-TGATGGCTC GGCGACCTG-3'), 298rcoIx3R (5'-ATAGTTGTCGGTATGGACAG-3'), 298rcoIx4R (5'-GATCGCGTGTCCACTCGC-3'), 298rcoIx1F (5'-AAGTGCAGCGAACTCAA GAC-3') and 298rcoIx2F (5'-GGGCTAAGGAGATGGGTTC-3'). Sequences were compiled to produce a 4584 kb sequence of the *rcoT* gene from *T. atroviride* LU298, which included the 2175 bp coding region and 1046 bp of sequence 5' to the ATG start codon.

Trichoderma hamatum

The *T. hamatum rcoT* sequence derived above was extended using inverse PCR essentially as described for *T. atroviride*. Inverse primers Irco1R and Irco1F were used to amplify 50 ng of *Kpn*I digested and self-ligated *T. hamatum* LU592 gDNA template. A resulting 5.5 kb inverse PCR product was cloned and sequenced using the T7 and SP6 promoters increasing the known sequence to ~1350 bp. The *Kpn*I clone was further sequenced using the *rcoT* specific primers 592rcoIx1R (5'-CGTCGTGGCTAAA GCGAAC-3'), 592rcoIx2R (5'-TTGAGCCATCTGGTGGTGG-3'), 592rcoIx3R (5'-

CTTTGTTTCAACTCCTGTGC-3'), rcoIx4R (5'-CTCAGGCGGTGATAAAATC-3'), 592rcoIx1F (5'-GGAGAACCGTTAATTAAGGAG-3') and 592rcoIx2F (5'-GAGAG AACCAAAGAGGCTGG-3'). Sequences were compiled to produce a 4473 bp sequence of the *rcoT* gene from *T. hamatum* LU592, which included the 2230 bp coding region and 997 bp of sequence 5' to the ATG start codon.

4.2.3.6 Identification of *rcoT* from the *Trichoderma reesei* Genome

A putative *rcoT* orthologue was identified in the *T. reesei* genome (Scaffold 43: 42940-50906 [http://genome.jgi-psf.org/Trire2/Trire2.home.html]) by conducting a Blastn search with the *T. atroviride rcoT* nucleotide sequence derived above. To confirm the identity of the search result, the putative *T. reesei rcoT* sequence was translated using the online software package Wise2 (http://www.ebi.ac.uk/Wise2) and the amino acid sequence aligned to all *Trichoderma* spp. sequences.

4.2.4 Isolation of *con-10*

An annotated putative *con-10* orthologue was identified in the *T. reesei* genome (Scaffold 19: 258000-259000 [http://genome.jgi-psf.org/Trire2/Trire2.home.html]) as described in 4.2.3.7 using the *Neurospora crassa con-10* sequence (ascn#: M20005) for blast searching. To confirm the identity of the search result, the putative *T. reesei con-10* sequence was translated as described in 4.2.3.7 and the amino acid sequence subjected to a Blastp search in GenBank (http://www.ncbi.nlm.gov/Genbank). Degenerate primers were designed (Figure 4.2) to regions of 100% identity in the protein sequence of *con-10* from *T. reesei* and *N. crassa* to amplify the *con-10* gene from *T. atroviride* (LU298) and *T. hamatum* (LU592) as described in 4.2.3.1. con10deg-1F (5'-GAYAAYCCNGGNAAYTTYGC-3') and con10deg-1R (5'-CNGCTY CNCKHGCYTTYTC-3') were predicted to amplify a 332 bp region 18 bp from the translational start site. Reaction components were as described in 4.2.3.2. Amplification consisted of an initial denaturation of 5 min at 94°C, followed by 60 cycles of 30 s at 94°C, 30 s at 65°C (decreasing by 0.5°C every cycle till cycle 30 then 50°C for remainder of cycles), and 30 s at 72°C, followed by a final extension of 10 min at 72°C. The resulting PCR product was cloned and sequenced in both directions as described in 4.2.3.3 and 4.2.3.4. To confirm identity, the sequences were aligned to the *T. reesei* and *N. crassa con-10* sequences using DNAman™ (Version 4.0, Lynnon Biosoft Corp.).

Amino Acid	[5] Asp Asn Pro Gly Asn Phe Ala [11]
Con10-5pF	5′ gaY aaY ccN ggN aaY ttY gc 3′

Amino Acid	[62] Glu Lys Ala Arg Glu Ala Gly [68]
Nucleotide (sense)	5′ gaR aaR gcN MgN gaR gcN ggN 3′
Con10-62pR (antisense)	3′ ctY ttY cgN KcN ctY cgN c 5′

Figure 4.2. *con-10* degenerate primer design. Blue represents the selected amino acid regions. Numbering is relative to the *T. reesei* Con-10 protein sequence. Degenerate primers are in red. Y = c/t, N = a/t/g/c, K = g/t.

4.2.5 Creation of *rcoT* Transformation Binary Vectors

4.2.5.1 *Trichoderma atroviride rcoT* Gene Replacement Vector

Construct creation

An overlapping PCR strategy was used to create a construct in which the majority of the coding region of *T. atroviride rcoT* was replaced by the *hph* (hygromycin phosphotransferase) gene (Figure 4.3 and Table 4.1). Primers were designed to amplify a 2364 bp region of the *hph* gene construct from the binary vector pYT6 (Appendix 7.5.2), kindly provided by Professor Barry Scott (Massey University), and to amplify regions 5' and 3' to the coding region of *rcoT* (left flank and right flank) from *T. atroviride* LU298. To facilitate joining of the three PCR products, tails complementary to the hphF and hphR primers were added to RatrLF-R and RatrRF-F respectively. Random sequence tails incorporating restriction enzyme sites were included in RatrLF-F and RatrRF-R for cloning of the construct into the binary vector.

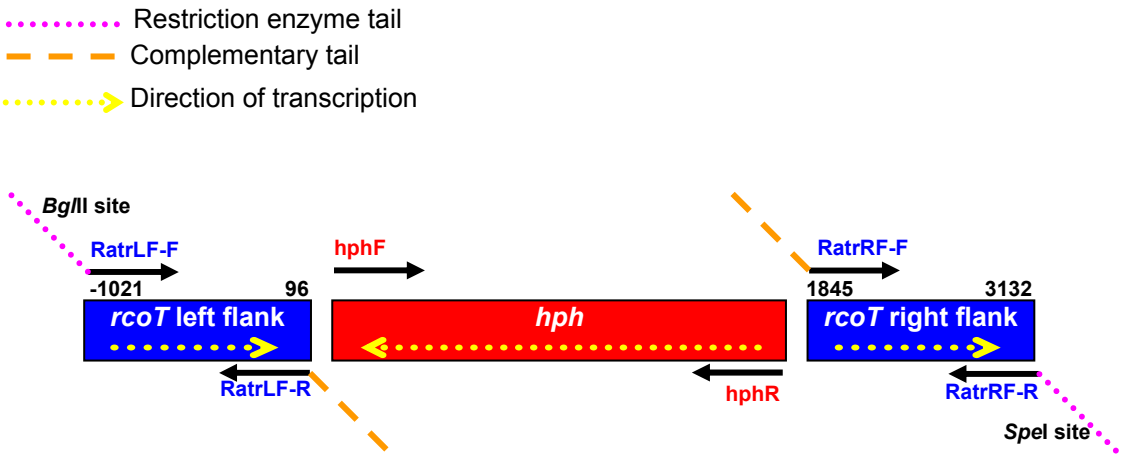


Figure 4.3. Overlapping PCR strategy for creation of the *T. atroviride* LU298 *rcoT* gene replacement construct. Numbering is relative to LU298 ATG at position 1.

In the initial PCR reactions the three fragments to be joined (Left Flank, Right Flank and HPH) were amplified separately. Each 50 µL reaction contained 1 X Expand Long Template buffer 2 (Roche Diagnostics GmbH.), 350 µM each of dATP, dCTP, dGTP and dTTP (Roche Diagnostics GmbH.), 40 pmoles of each primer, 40 ng of LU298 gDNA or 2 ng pYT6 and 0.75 µL Expand Long Template Enzyme mix (3.75 U). Amplification consisted of an initial denaturation of 2 min at 94°C, followed by 20 cycles of 10 s at 94°C, 30 s at 64°C and 2 min at 68°C, followed by a final extension of 7 min at 68°C. A 10 µL aliquot of each reaction was size fractionated by 1% agarose gel electrophoresis as described in Appendix 7.2.5. The remaining PCR products were reprecipitated as described in 2.4.2.5 and unincorporated primers and dNTPs removed using Quantum Prep® PCR Kleen Spin Columns (Bio-Rad Laboratories Inc.) as per manufacturer's instructions. The purified fragments were quantified by gel electrophoresis as described in Appendix 7.2.5.

Equimolar amounts of the three fragments, Left Flank, Right Flank and HPH, were used as template in a second PCR reaction with primers RatrLF-F and RatrRF-R to amplify a predicted 4.9 kb complete *rcoT* gene disruption construct. Reaction components were as described above except that 40 ng each of Left Flank and Right Flank and 20 ng of HPH was used as template. Amplification consisted of an initial denaturation of 2 min at 94°C, followed by 15 cycles of 10 s at 94°C, 30 s at 66°C and 5 min at 68°C, followed by a final extension of 7 min at 68°C. The resulting PCR products were size fractionated by 0.8% gel electrophoresis and a 4.9 kb fragment purified from agarose using the QIAEX II Gel Extraction Kit (QIAGEN Pty. Ltd.) according to manufacturer's instructions. The complete construct was stored at -20°C.

Insertion of Construct into the Binary Vector

The *T. atroviride rcoT* gene replacement construct was cloned into a pCAMBIA binary vector in which the *hph* gene had been removed. A 1 µg aliquot of pCAMBIA 1380 was digested to completion using *Xho*I and *Sal*I (Roche Diagnostics GmbH.), according to manufacturer's instructions, in a total volume of 25 µL. The resulting 7099 bp fragment was purified from 0.8% agarose as described above, re-ligated by 2 U T4 DNA ligase (Roche Diagnostics GmbH.), according to manufacturer's instructions, in a total volume of 20 µL and then transformed into *E. coli* strain INVαF' (Invitrogen Corp.) as described in 4.2.3.3, except that 50 µg kanamycin (Sigma-Aldrich NZ Ltd., Auckland, New Zealand) was used for selection instead of ampicillin. Plasmid DNA was prepared

using the Wizard® Plasmid Mini kit (Promega Corp.) as per manufacturer's instructions. A 500 ng aliquot of the modified binary vector (p1380XS) and the eluted construct from above (20 µL) were digested separately to completion with *Bgl*II and *Spe*I (Roche Diagnostics GmbH.) in a total volume of 30 µL. The vector and construct fragments were purified from 0.8% agarose as described above and then quantified by gel electrophoresis as described in Appendix 7.2.5. The construct was ligated to the vector at a 3:1 construct to vector molar ratio, using 50 ng of DNA in total, with 1 U T4 DNA ligase (Roche Diagnostics GmbH.), according to manufacturer's instructions, in a total volume of 25 µL. Ligated plasmids were transformed into *E. coli* strain INVαF' (Invitrogen Corp.) and plasmid DNA from 12 clones per transformation was prepared as described above. Plasmid DNA (100 ng) from each of the clones was digested to completion with *Bgl*II and *Spe*I (Roche Diagnostics GmbH.) in a total volume of 15 µL and size fractionated by gel electrophoresis to confirm presence of the construct within the vector.

Vector Sequencing

The completed transformation vector created above was sequenced across overlapping PCR joins and vector/insert boundaries to confirm the construction was as predicted, and the vector designated pRx298. Sequencing primers were specific to the *rcoT* regions within pRx298. Ratr112-R (5'-GTATCTGCTGAGGATTCTG-3') was designed to sequence the pCAMBIA/Left Flank boundary, Ratr1012-F (5'-CTTG AATCCCACAGTCGC-3') the Left Flank/HPH boundary, Ratr3412-R (5'-TCGG TCATATGACTCTCTG-3') the HPH/ Right Flank boundary and Ratr4311-F (5'-AGAAGGCCCTGTGTATGAG-3') the Right Flank/pCAMBIA boundary. pRx298 was sequenced directly with these primers at the Canterbury DNA Sequencing Facility (Canterbury University).

4.2.5.2 *Trichoderma hamatum rcoT* Gene Replacement Vector

A *T. hamatum rcoT* gene replacement construct was created essentially as described for *T. atroviride* above (Table 4.1). The Left Flank was amplified using RhamLF-F and RhamLF-R and an annealing temperature of 62°C, and the Right Flank was amplified using RhamRF-F and RhamRF-R and an annealing temperature of 60°C. A 4.6 kb complete construct was amplified in a second PCR reaction using RhamLF-F and RhamRF-R and an annealing temperature of 64°C. The construct was purified from agarose, then digested with *Bgl*II and *Spe*I (Roche Diagnostics GmbH.) and ligated to

the modified binary vector p1380XS and transformed to *E. coli* as described in 4.2.3.3. The vector was sequenced directly to confirm identity and designated pRx592. Rham205-R (5'-CTAGCCTGGCTGCAACTG-3') was designed to sequence the pCAMBIA/Left Flank boundary, Rham910-F (5'-GAATCGAGTCGAATCGATTG-3') the Left Flank/HPH boundary, Rham3502-R (5'-GAGCAAGAACTGGACTTGG-3') the HPH/Right Flank boundary and Rham4251-F (5'-GATTGTCGCTTGAGAGGAG-3') the Right Flank/pCAMBIA boundary.

4.2.5.3 *Trichoderma atroviride rcoT* Antisense Vector

A *T. atroviride rcoT* construct with exons 3 and 4 placed under the control of a constitutive promoter in the antisense direction was created and cloned into the binary vector pYT6 (Appendix 7.5.2). Using an overlapping PCR strategy the *GlaA* promoter (GLA) and the *TrpC* terminator (TRP) from pYT6 were joined to the inverted exons 3 and 4 (Rex4-3) (Figure 4.4, Table 4.1). To facilitate joining of the three PCR products primers gla-R and trp-F had tails complementary to Rex4AS-F and Rex3AS-R, respectively. Fragments were amplified separately as described in section 4.2.5.1 except that an annealing temperature of 56°C was used for primer set Rex4AS-F/Rex3AS-R. Equimolar amounts of the three fragments were used as template in a second PCR reaction with primers gla-F and trp-R to amplify a predicted 3 kb complete *rcoT* antisense construct as described in 4.2.5.1 except an annealing temperature of 60°C was used. The purified construct was restriction digested with HindIII (Roche Diagnostics GmbH.) and ligated into pYT6 at a molar ratio of 1:2 vector to construct. The construct was transformed into *E. coli* as described in 4.2.5.1. The cloned vector was sequenced directly to confirm identity and designated pRAS. RB-1R (5'-TATT GCCAAATGTTTGAACG-3') was specific to pYT6 between the right border and *Hind*III site and designed to sequence the pYT6/*GlaA* and *GlaA*/*rcoT* boundaries and RAS3'F (5'-CATGATGCCGCTGAAGAG-3') the *rcoT*/*TrpC* boundary. The *TrpC*/pYT6 boundary region was amplified directly from pRAS using Trp392F (5'-CCTACGAGACTGAGGAATCC-3') and Gla114R (5'-GGAAGCTATAGTGCA GGTCC-3'). Reaction conditions and amplifications were as described in 4.2.3.3. The resulting PCR product was sequenced directly using Trp392F.

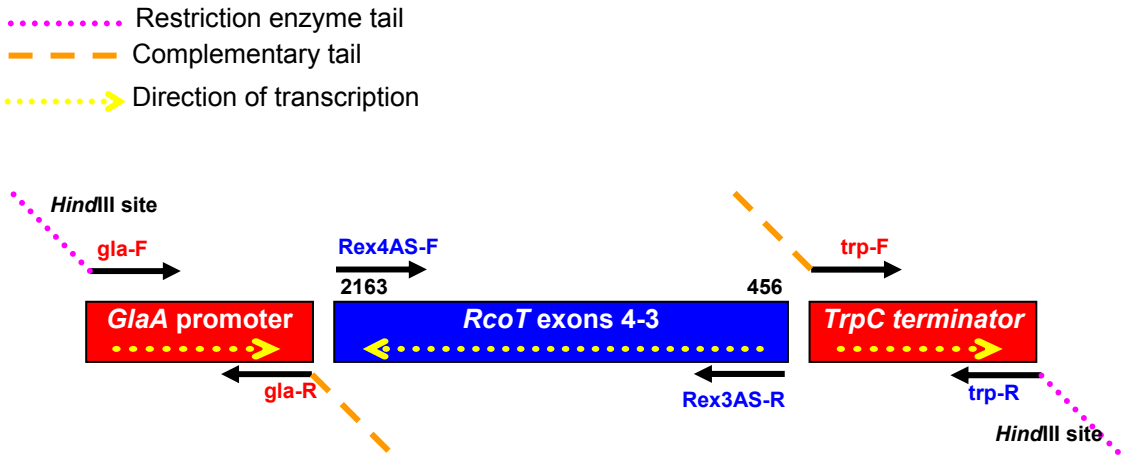


Figure 4.4. Overlapping PCR strategy for creation of the *T. atroviride* LU298 *rcoT* antisense construct. Numbering is relative to LU298 ATG at position.

Table 4.1. Primers used in creation of transforming constructs. Red = *hph* specific, blue = *rcoT* specific, green = random with restriction enzyme site inserted. Restriction enzyme sites are underlined.

Primer	Sequence
hphF	CTTATCTGGGAACTACTCACAC
hphR	AGGGTATATACCACGCGTTGG
RatrLF-F	ATATAGATCTAACTGCTCAAAACCCTCCTAGGC
RatrLF-R	GTGTGAGTAGTTCCCAGATAAGCTGTCGAACTCGGCTCTAATC
RatrRF-F	CCAACGCGTGGTATATACCCTGACAGGAGTGGTAGGACGCC
RatrRF-R	TATAACTAGTTTGCAACCTCATTGACGATCTCTC
RhamLF-F	ATATAGATCTAATCCTAGTCTGCCGTACATACC
RhamLF-R	GTGTGAGTAGTTCCCAGATAAGCTCAAAGCTCTCGGTTTGACG
RhamRF-F	CCAACGCGTGGTATATACCCTAAGGAGAACCGTTAATTAAGGAG
RhamRF-R	TATAACTAGTTTGTTCAATCTCTACCTATGCCTC
GlaF	ATATAAGCTTAAACCATGGTGGACTCCTCTTAAAG
GlaR	GAAGGCTCGTATCTGGTCGTATGAGTACCGATGTTGAGGATGAAG
Rex4AS-F	ATACGACCAGATACGAGCCTTC
Rex3AS-R	ATATGAGGAGGAAATCAACATGCT
TrpF	AGCATGTTGATTTCCTCCTCATATGATCGATCCACTTAACGTTACTGA
TrpR	ATTAAGCTTATTTCGACTCTAGAAAGAAGGATTACC

4.2.6 Transformation of Binary Vectors into *Agrobacterium tumefaciens*

4.2.6.1 Preparation of *A. tumefaciens* Competent Cells

Electro-competent cells of *A. tumefaciens* strain EHA105 were prepared using standard laboratory techniques. A single colony of EHA105 was inoculated to 30 mL LB broth amended with 25 µg/mL Rifampicin (Appendix 7.2.6) and grown overnight at 30°C, shaking at 250 rpm. In the morning 5 mL was inoculated into 2 X 500 mL Yeast Mannitol (YM) broth (Appendix 7.2.6) in 1 L conical flasks and incubated overnight at 30°C, shaking at 300 RPM. Aliquots of 45 mL were centrifugated at 3000 x*g* for 10 min at 4°C in 50 mL Nunc tubes (Nalge Nunc International Inc.) and the cell pellet resuspended by vortexing in 40 mL ice-cold 10% glycerol. Cells were pelleted again as described and resuspended by gentle inversion in 40 mL ice-cold 10% glycerol. Cells were centrifuged as described above and gently resuspended in 500 µL ice-cold 10% glycerol and 10 tubes combined. Cells were pelleted once more as described and the pellets resuspended in 500 µL ice-cold 1 M sorbitol. Aliquots of 40 µL were transferred on ice to 1.5 mL centrifuge tubes and stored at –80°C till required.

4.2.6.2 Electroporation of *A. tumefaciens*

The binary vectors created in section 4.2.5 were electroporated to *A. tumefaciens* EHA105 using standard laboratory techniques. For each sample to be electroporated 5µL of binary vector DNA in a 1.5 mL centrifuge tube and a 0.1 cm electroporation cuvette were placed on ice. Competent cells were thawed on ice and 20 µL added to each DNA sample and mixed by gently tapping the tubes. The DNA/cell mix was transferred to the electroporation cuvette and the suspension gently tapped to the bottom of the tube. The cuvette was placed in the chamber slide and the slide placed in the chamber and pulsed once using the "Agr" setting (2.5 KV, 25 uF, 200 ohms, 4-5 msec pulse) on a Bio-Rad Micropulser (Bio-Rad Laboratories Inc.). The cells were immediately transferred to a 15 mL Nunc tube (Nalge International Inc., Naperville, IL, USA) using 1 mL YM broth (Appendix 7.2.6) and incubated for 3 h at 30°C, shaking at 250 RPM. For each transformation 2 X 50 µL aliquots of the electroporated cells were plated to YM agar plates amended with 25 µg/mL rifampicin and kanamycin (Appendix 7.2.6) and incubated for 48 h at 30°C.

4.2.6.3 Confirmation of Transformation

Presence of the binary vector with *A. tumefaciens* was confirmed through restriction digestion. Plasmid DNA from 3 clones per transformation was prepared using the

Wizard® Plasmid Mini kit (Promega Corp.) as per manufacturer's instructions. For each clone 2 µg of plasmid DNA was digested with either *Bgl*II and *Spe*I (pRx298 and pRx592) or *Hind*III (pRAS) (Roche Diagnostics GmbH.) according to manufacturer's instructions and size fractionated by 0.8% gel electrophoresis.

4.2.7 *Agrobacterium*-mediated Transformation of *Trichoderma*

Background sensitivity of *T. atroviride* LU298 and *T. hamatum* LU592 was determined using PDA amended with increasing levels of Hygromycin-B (Invitrogen Pty. Ltd.). In the first experiment the PDA was sterilised in a laboratory bench top pressure cooker, allowed to cool to 50°C and then amended with Hygromycin-B at 125, 150, 175 and 200 µg/mL. Four plates per isolate were inoculated centrally with 2 µL of a 10^8 conidial suspension, previously stored at -80°C in 20% glycerol (Appendix 7.2.2), and incubated unsealed at 23°C for 7 d in total darkness. In the second and third experiments, LU298 only was inoculated to PDA, acidified to pH 4.0 prior to sterilisation and then amended with either 125 to 225 µg or 225 to 475 µg Hygromycin-B, with intervals of 25 µg.

Using the binary vectors created above, *T. atroviride* LU298 and *T. hamatum* LU592 were transformed via *A. tumefaciens* essentially as described in Zeilinger *et al* (2004) and Zwiers and De Waard (2001), with modifications. *Agrobacterium tumefaciens* strains were prepared as follows: a single colony of EH105 transformed with either pRx298, pRx592 or pRAS was inoculated to LB broth amended with 25 µg/mL each of rifampicin and kanamycin and incubated overnight at 28°C, with shaking at 250 RPM. Cultures were diluted 1:100 in *A. tumefaciens* minimal media (AtMM) (Zwiers and De Waard, 2001) with selection and incubated overnight again as described above. Cells were then collected by centrifugation at 3000 x*g* for 10 min at room temperature and resuspended in acetosyringone induction-medium (IM) (Zwiers & De Waard, 2001) to an optical density of 0.15 at 660 nm and incubated at 28°C, with shaking at 70 RPM, until an optical density of 0.3 at 660 nm. Prepared cells were mixed at a 1:1 ratio with either LU298 or LU592 conidia suspended in 0.9 M NaCl at 10^7/mL and 200 µL aliquots of the mix was plated onto sterile cellophane disks placed on induction-medium. Bacterial cells and conidia were co-cultivated at 23°C for 1 or 2 days before the cellophane disks were transferred to PDA amended with 300 µg/mL each of Hygromycin B (Invitrogen Pty. Ltd.) and Timentin (Appendix 7.2.6) and covered with a 3 mL overlay of 0.7% agarose with the same selection. Plates were incubated at 25°C in the dark and colonies which appeared were transferred to PDA with hygromycin B and

timentin. For the antisense experiments pH 4.0 unbuffered PDA (4PDA) and 600 µg/mL hygromycin B was used.

4.2.8 Purification of Transformants

To obtain monokaryotic transformants, hygromycinB-resistant, putative transformants were allowed to conidiate on 4PDA (Appendix 7.2.3) amended with 600 µg Hygromycin B (Invitrogen PTY. Ltd.) and single spore isolation was performed consecutively three times followed by once with no selection. To confirm stability, transformants were subcultured to non-selective medium and allowed to grow across the plate in total darkness three times then subcultured back to selection plates. Spores from all transformants were stored in 20% glycerol at -80°C.

4.2.9 PCR Analysis of *rcoT* Transformants

4.2.9.1 Gene Replacement Experiments

4.2.9.1.1 *Confirmation of Homologous Recombination*

To test for homologous recombination, primers were designed to a region upstream of the predicted homologous integration points of the LU298 and LU592 *rcoT* gene disruption constructs and to a region within the *hph* gene. PCR using these primers was predicted to generate amplimers of 1840 and 1728 bp, respectively. Primer set Ratr5'-1F (5'-CTCTACAACGTTGAGAGGAC-3') and Hph-2R (5'-TCAAGAGA CCTACGAGACTG-3') was used to test for recombination in Rx298 transformants and primer set Rham5'-1F (5'-CCTAATCTCCTTGCATGTGC-3') and Hph-2R was used with the Rx592 transformants. Reaction components were as described in 4.2.3.2 except 10 pmoles of each primer was used. gDNA from either *T. atroviride* LU298 or *T. hamatum* LU592 was included as a control in all PCR reactions. Amplification consisted of an initial denaturation of 5 min at 94°C, followed by 60 cycles of 30 s at 94°C, 30 s at 60°C, and 2 min at 72°C, followed by a final extension of 10 min at 72°C. Resulting PCR products were size fractionated by 1% gel electrophoresis as described in Appendix 7.2.5.

4.2.9.1.2 *Nested PCR Confirmation of Homologous Recombination*

To increase detection sensitivity, a nested PCR strategy was designed and used to test for homologous recombination in Rx298 and Rx592 transformants. The PCR reactions described in 4.2.9.1.1 were diluted 1:200 with sterile water and 1 µL used as template in a secondary PCR with primers designed within the primary PCR amplimer. Primer set

Ratr5'-2F (5'-AAGGTCAAGAATGTGTCATCC-3') and Hph-3R (5'-TGAACTCTCAAGCCTACAGG-3') was predicted to amplify 1596 bp from the Rx298 primary PCR products and primer set Rham5'-2F (5'-GCATGTGTATGGTGCAGTGT-3') and Hph-3R was predicted to amplify 1533 bp from the Rx592 primary PCR products. PCR reactions and amplifications were as described in 4.2.9.1.1. Resulting PCR products were size fractionated by 1% gel electrophoresis as described in Appendix 7.2.5.

4.2.9.1.3 *Amplification of* rcoT *– Negative Control*

As a negative control for the homologous recombination PCR analysis, primers RAtr156F (5'-CATACCGACAACTATTGCGC-3') and RAtr533R (5'-CCTTGCAG GCCAGACTGC-3') were designed and used to amplify a 378 bp portion of *rcoT* predicted to be absent. Reaction conditions and amplifications were as described in 4.2.9.1, except an extension time of 30 s was used. Resulting PCR products were size fractionated by 1% gel electrophoresis as described in Appendix 7.2.5.

4.2.9.1.4 *Amplification of the* hph *Gene*

To confirm the presence of the gene replacement construct, primer hph-2F (5'-AGTGTACCTGTGCATTCTGG-3') was designed and used with primer hph-2R (4.2.9.1.1) to amplify a predicted ~ 350 bp region of the *hph* gene from putative *rcoT* gene replacement transformants. Reaction conditions and amplifications were as described in 4.2.9.1, except an extension time of 30 s was used. Resulting PCR products were size fractionated by 1% gel electrophoresis as described in Appendix 7.2.5.

4.2.9.1.5 *Nested Amplification of* hph *Gene*

To increase detection sensitivity, a nested PCR strategy was designed to amplify the *hph* gene from putative transformants. Primers hphF and hphR were designed in section 4.2.5 to amplify an ~ 2360 bp region of the *hph* gene and were used in the primary PCR reactions. Reaction conditions and amplifications were as described in 4.2.9.1, except an annealing temperature of 64°C and an extension time of 2.5 min were used. Primary PCR reactions were diluted 1:200 with sterile water and 1 µL used as template in a secondary PCR with primers designed previously within our laboratory to amplify an ~600 bp region of the *hph* gene (Dr Richard Weld, pers comm.). Reaction conditions and amplification were as described in 4.2.9.1.1, except an extension time of 30 s was

used. Resulting PCR products were size fractionated by 1% gel electrophoresis as described in Appendix 7.2.5.

4.2.9.2 Antisense Experiments

Primers hpha and hphb (described in 4.2.9.1.5) were used to confirm the presence of the *hph* gene in gDNA from all putative antisense transformants. Reaction conditions and amplification were as described in 4.2.9.1.1, except an extension time of 30 s was used. Resulting PCR products were size fractionated by 1% gel electrophoresis as described in Appendix 7.2.5.

4.2.10 Southern Analysis of Antisense Transformants

4.2.10.1 DNA Preparation

An agarose gel containing digested gDNA from LU298 and all antisense transformants and pRAS plasmid DNA was prepared for Southern transfer. For each isolate 5 µg of gDNA was digested to completion with *Bst*XI (Roche Diagnostics GmbH.) according to manufacturer's instructions and then reprecipitated and re-dissolved in 20 µL H_2O as described in 4.2.3.5. Digested DNA was size fractionated by 0.8% gel electrophoresis as described in 7.2.5. The gel was rinsed in distilled H_2O then submerged in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and agitated for 2 X 15 min, followed by another rinse in distilled H_2O. The gel was then submerged in neutralisation solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 2 X 15 min gentle agitation, then transferred to 20X SSC (Appendix 7.2.7) and allowed to equilibrate for 10 min.

4.2.10.2 Capillary Transfer

A gel casting tray was placed upside down in a large pyrex dish and transfer buffer, 20X SSC, added till just beneath the tray. A wick made from 3 pieces of 3MM paper soaked in transfer buffer was placed on top of the tray. The agarose gel was placed directly on to the wick and parafilm arranged 2-3 mm under the edges of gel. A section of Hybond-N+ nylon membrane (Amersham Pharmacia Biotech UK Ltd.) was cut to size was carefully placed on the gel avoiding airbubbles and this was covered with 3 X 3MM paper cut to size and soaked in transfer buffer. A 5 cm stack of paper towels was placed on 3MM paper, followed by a glass plate and a weight of 750 g. Capillary transfer was allowed to proceed overnight and in the morning the transfer apparatus was disassembled, the position of the wells marked on the blot with a chinograph pencil and the membrane was baked for 2 h at 80°C to fix the nucleic acid.

4.2.10.3 DIG Chemiluminescent Detection of *hph* DNA

4.2.10.3.1 hph *Probe*

Plasmid DNA containing a portion of the *hph* gene was used to generate a probe to detect construct DNA on the Southern blot. Using primers HphCod-F (5'-GATGTAGGAGGGCGTGGATA-3') and HphCod-R (5'-GATGTTGGCGACCT CGTATT-3') (designed previously, Dr Margaret Carpenter, pers. comm.) and pYT6 an ~600 bp region of the *hph* gene was labelled using the DIG PCR Probe Synthesis Kit (Roche Diagnostics GmbH.) with an annealing temperature of 60°C, according to manufacturers instructions.

4.2.10.3.2 *Chemiluminescent Detection*

Chemiluminescent detection of DNA was performed using the DIG Luminescent Detection Kit (Roche Diagnostics GmbH) according to manufacturer's instructions. For each reaction, 250 ng of DIG-labelled DNA was hybridised in tubes (Thermo Hybaid) overnight to the immobilised DNA in 20 mL DIG Easy Hyb. Blots encased in mylar sheets were placed in an X-ray film cassette and a sheet of Kodak BioMax XAR Film (Kodak [Australia] Pty., Ltd.) placed on top. The cassette was closed and the film exposed for 3 min. Films were developed manually using the Kodak GBX system (Kodak [Australia] Pty., Ltd.).

4.2.11 Analysis of Gene Expression in Antisense Transformants

4.2.11.1 RNA Extraction

Total RNA was extracted from LU298 and antisense transformants grown in total darkness for analysis of gene expression. Inoculum plugs from 2 day old PDA cultures grown on PDA at 23°C in total darkness were inoculated to cellophane which was placed on PDA and incubated for 3 days at 23°C in total darkness. Whole colonies were harvested by snap freezing in liquid nitrogen and stored at -80°C. Total RNA was extracted from frozen mycelium using the QIAGEN RNeasy® Plant mini Kit (QIAGEN Pty. Ltd.) as per manufacturer's instructions for fungi. Each sample was ground to a fine powder in a mortar and pestle with liquid nitrogen and 100 mg used as starting material in the RNA extraction kit. All samples were stored for up to 1 week at -20°C and indefinitely at -80°C. RNA concentrations were determined spectrophotometrically using a NanoDrop® ND-1000 (NanaDrop Technologies Inc.) and where the concentration was below 1500 ng per µL samples were reprecipitated and dissolved in a lesser volume as described by Sambrook *et al.* (1989).

4.2.11.2 Preparation of the Dot Blots

To prepare the Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories Inc.), its components were washed in hot water and allowed to air-dry, then UV-irradiated to remove residual RNA. A section of Hybond-N+ nylon membrane (Amersham Pharmacia Biotech UK Ltd.) at least one well wider than the samples to be blotted was pre-wetted in 5 x SSC (Appendix 7.2.7) and the Bio-Dot was assembled according to manufacturer's instructions. Wells not in use were covered with scotch tape. To each sample well, 100 μL of H_2O was added, and transferred through the membrane by gentle vacuum.

Three dot blots were prepared using the RNA extracted in 4.2.11.1. In the first blot 20 μg of RNA from LU298 and each antisense transformant was applied to the membrane. the remaining two blots were prepared together and 10 μg of each sample was applied. RNA was denatured by the addition of 500 μL of ice-cold 10 mM NaOH, 1 mM EDTA. Samples were applied immediately to the Biodot wells using a gentle vacuum. Individual wells were covered with scotch tape once the solution had drained to avoid damaging the membrane through excessive vacuum pressure. The tape was removed and a further 500 μL of ice-cold denaturation solution applied to wash RNA from the well walls. The tape was removed and maximum vacuum applied until all wells were dry. The Bio-Dot was disassembled and the membrane rinsed briefly in 2 x SSC, 0.1% SDS. Blots were air-dried on 3MM Whatman filter paper and then oven baked at 80°C for 2 h to fix the RNA.

4.2.11.3 DIG Chemiluminescent Detection of *rcoT*: Dot Blots

4.2.11.3.1 *Detection of Sense and Antisense* rcoT *with a DNA Probe*

Using the DIG PCR Probe Synthesis Kit (Roche Diagnostics GmbH.), a DNA probe of *rcoT* was created to detect mRNA on the first RNA dot blot (4.2.11.2). A portion of the *rcoT* gene spanning exon 3-4 was cloned from LU298 and used to generate a probe to detect sense and antisense *rcoT* mRNA on the dot blot. Using primers RProbeF (5'-ATGTCGACCTGGTCCACTC-3') and RprobeR (5'-GGGCAACTGAGA TGACAGAG-3') a 950 bp region of *rcoT* was amplified and cloned as described in 4.2.3.3, except an annealing temperature of 60°C was used. This clone (pRcoPr-1) was used as template in a PCR labelling reaction with primers RProbeF/RProbeR to generate a labelled probe for chemiluminescent detection, according to manufacturer's instructions (DIG PCR Probe Synthesis Kit [Roche Diagnostics GmbH.]).

Chemiluminescent detection of RNA was performed using the DIG Luminescent Detection Kit (Roche Diagnostics GmbH), according to manufacturer's instructions and recommendations for probing RNA blots with DNA probes. For each reaction, 250 ng of DIG-labelled DNA was hybridised in tubes (Thermo Hybaid, Ashford, Middlesex, UK) overnight to the immobilised RNA in 10 mL DIG Easy Hyb. Blots encased in mylar sheets were placed in an X-ray film cassette and a sheet of Kodak BioMax XAR Film (Kodak [Australia] Pty. Ltd.) placed on top. The cassette was closed and the film exposed for 3 min. Films were developed manually using the Kodak GBX system (Kodak [Australia] Pty., Ltd.).

4.2.11.3.2 *Detection of Sense and Antisense* rcoT *with RNA probes*

Using the DIG RNA Labelling Kit (Roche Diagnostics GmbH.), RNA probes were created to detect sense (wt) *rcoT* mRNA on blot 2 and antisense (mutant) *rcoT* mRNA on blot 3. The sense probe was created as follows: approximately 2.5 µg of the pRcoPr-1 clone, created above, was digested to completion at the *Not*I (Roche Diagnostics GmbH.) site in the pGEM®-T backbone (Appendix 7.3.1). The restriction enzyme and salts were removed using the QIAEX II Gel Extraction Kit (QIAGEN Pty. Ltd.) and following the manufacturer's recommendations for desalting and concentrating DNA from solutions. The DNA was then precipitated and dissolved in 10 µL H_20, as described in 4.3.2.5. Using the DIG RNA Labelling Kit (Roche Diagnostics GmbH.), approximately 1 µg of the purified, linearised plasmid was used as template in a transcriptional labelling reaction using the T7 RNA Polymerase, according to manufacturer's instructions. Approximately 1 µg of the labelled *rcoT* RNA probe was used to detect sense mRNA as described in 4.2.11.3.1, using manufacturer's recommendations for probing RNA blots with RNA probes (DIG Luminescent Detection Kit [Roche Diagnostics GmbH.]).

The antisense probe was created using a PCR product generated from the pRcoPr-1 clone as template for the labelling reactions. Primer T3pGEMT (5'-CATAATTAACCCTCACTAAAGGGCTCAAGCTATGCATCCAACG-3') was designed to pGEM®-T between the SP6 promoter and the TA-cloning sites and contained a T3 promoter sequence tag (underlined region). Using primer T3pGEMT and a second primer designed to the T7 promoter site, approximately 1 kb of pRcoPr-1 containing the full *rcoT* insert was amplified using the Expand Long Template PCR System (Roche Diagnostics GmbH.), as per manufacturer's instructions. Using the DIG

RNA Labelling Kit (Roche Diagnostics GmbH.), a 4 µL aliquot of the PCR reaction was used as template in a transcriptional labelling reaction using the T3 RNA Polymerase, according to manufacturer's instructions. Approximately 1 µg of the labelled *rcoT* RNA antisense-probe was used to detect sense mRNA as described for the sense probe.

4.2.11.4 Preparation of the Northern Blot

RNA from LU298 and all antisense transformants (4.2.11.1) was size fractionated in a Northern gel and transferred to a nitrocellulose membrane using standard laboratory techniques (Sambrook *et al*., 1989). A 1% agarose gel was prepared by dissolving 2 g Agarose in 20 mL 10 X MOPS buffer (Appendix 7.2.7) and 169 mL H_2O, cooling it to 50°C and adding 11 mL formaldehyde before pouring into a 20 x 8 cm gel casting tray containing a well-forming comb and allowed to set for ~45 min. The comb was removed and the casting tray containing the solidified gel transferred to an electrophoresis tank (E-C Apparatus Corp.) containing 1 X MOPS buffer. A 10 µL aliquot of each sample containing approximately 15 µg total RNA was mixed with 28 µL RNA sample buffer (Appendix 7.2.7) and heated to 65°C for 15 min then placed on ice for 5 min. RNA loading dye (4 µL) and 2 µL 1 mg/mL ethdium bromide were added to the denatured RNA and samples were loaded to the prepared gel, which had been pre-run for 5 min. The RNA was size fractionated by electrophoresis at a constant voltage (12.5 V/cm) for 1 h. The gel was then photographed using a Versadoc Imaging System Model 3000 (Bio-Rad Laboratories Inc.) and washed 3 X 10 min in 10 X SSX. RNA was transferred and fixed to nylon membrane as described in 4.2.10.2.

4.2.11.5 DIG Chemiluminescent detection of *rcoT* and *con-10*: Northern Blot

Using the DIG PCR Probe Synthesis Kit and DIG Luminescent Detection Kit (Roche Diagnostics GmbH.), DNA probes of *rcoT* and *con-10* were used to detect mRNA on the northern blot. The labelled *rcoT* DNA probe was prepared as described in 4.2.11.3.1. Using primers con10deg-1F and con10deg-1R, a labelled probe was generated from the *con-10* clone created in 4.2.5. PCR labelling was done according to manufacturer's instructions, using an annealing temperature of 55°C. Chemiluminescent detection was done as described in 4.2.11.3.1 and 4.2.10.3.2.

4.2.12 Phenotypic Analysis of Antisense Mutants

4.2.12.1 Evaluation of Colony Extension Rate

Growth rates in total darkness were evaluated on PDA for *T. atroviride* LU298 and all antisense transformants. The plates were prepared and inoculated as described in 4.2.7 and incubated under total darkness at 20°C for 4 days. The colony diameter was measured under safe red light at 24, 36, 48 and 60 h and colony growth calculated on the basis of extension from 24 h onwards. Five plates per isolate were inoculated and the experiment was repeated once.

4.2.12.2 Incubation under Alternating Light/Dark Conditions

The effect of alternating light/dark exposure on conidiation was examined on PDA medium for the *T. atroviride* wild-type, LU298, and all antisense transformants. The PDA was prepared and inoculated as described in 4.2.7 and plates were incubated unsealed at 23°C under 12/12 h alternating light/dark cycles, with standard white light, for 5 d. Three plates per isolate were inoculated and the experiment was not repeated.

4.3 Results

4.3.1 Sequence Analysis of *rcoT*

4.3.1.1 Isolation of *rcoT* from *Trichoderma* spp.

Using a combination of degenerate, inverse and specific PCR, *rcoT*, an orthologue of *rco1* (*N. crassa*) and *tupA* (*A. nidulans*), was cloned and sequenced from *T. atroviride* LU298 and *T. hamatum* LU592. Using primers Rco384pS and Rco554pAS a single band of 470 bp was amplified and sequenced from both isolates revealing ~61% nucleotide identity and ~82% amino acid identity to *N. crassa rco1* (ascn: U57061). Multiple inverse PCR clones were used to extend the 470 bp *rcoT* sequences to 5171 bp for *T. atroviride* and 5150 bp for *T. hamatum* LU592. The combined *T. atroviride* sequence was composed of 1445 bp 5' to the translational start site, the complete 2175 bp coding region and 1551 bp 3' to the stop codon. The *T. hamatum rcoT* sequence was composed of 1315 bp 5' to the start codon, the complete 2230 bp coding region and 1605 bp 3' to the stop codon. A predicted 614 and 615 amino acid sequence for *T. atroviride* and *T. hamatum*, respectively, was derived using the Wise2 program (http://www.ebi.ac.uk/Wise2).

Subsequent to the sequencing of *rcoT* from *T. atroviride* and *T. hamatum* the *T. reesei* genome became publicly available enabling the identification of the annotated *rcoT* orthologue. The amino acid sequence of *T. atroviride rcoT* was subjected to a blastn search within the *T. reesei* genome to reveal an annotated putative Rco1 orthologue (scaffold 9: 1169397 to 1172370 [http://genome.jgi-psf.org/Trire2/Trire2.home.html]). Alignment of coding regions and amino acid sequences revealed a high level of similarity between the three *Trichoderma* species and between *T. atroviride* and *rco1* orthologues from other filamentous fungi. (Figure 4.5A & B). A nucleotide sequence alignment of *rcoT* from *T. atroviride* and *T. hamatum* is presented in Appendix 7.4.3.

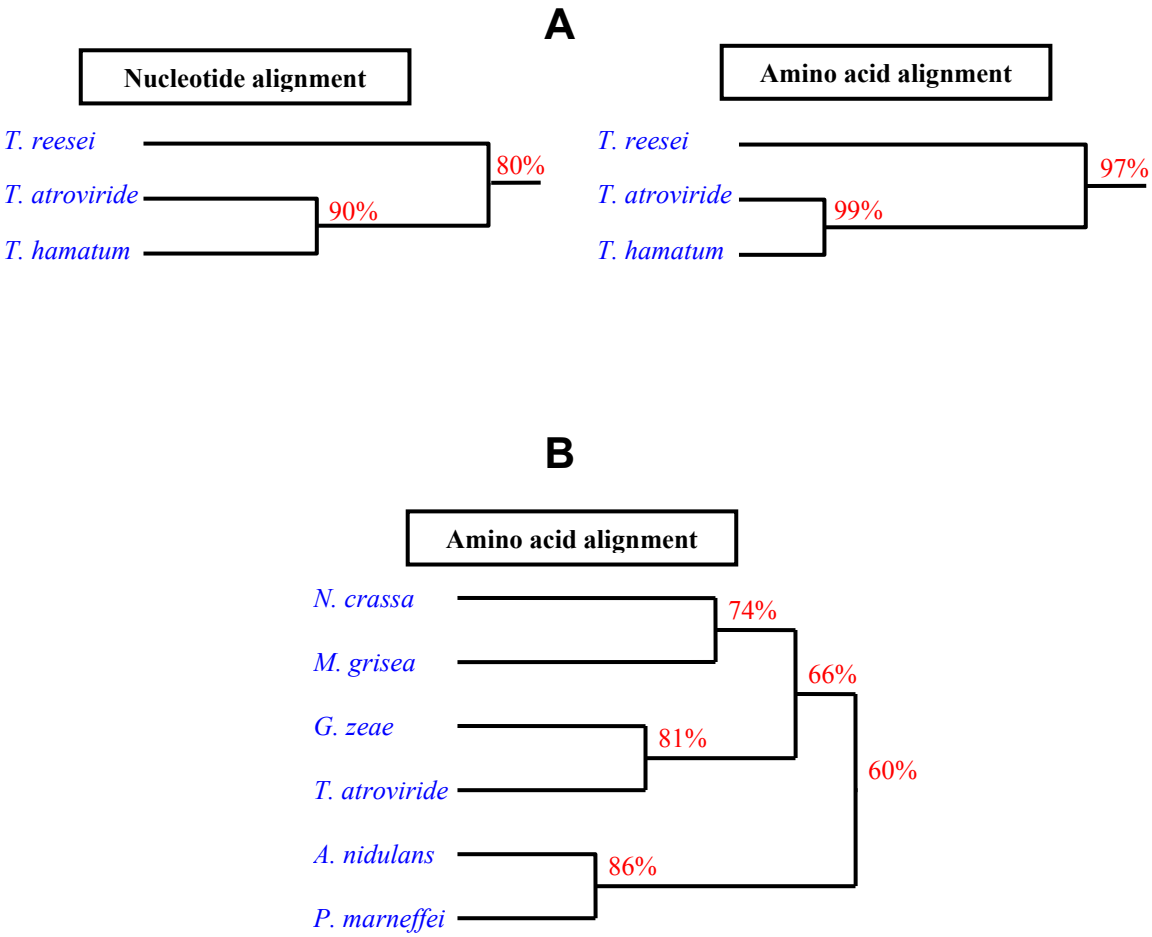


Figure 4.5. Homology trees based on sequence alignments of *rco1* orthologues. **A.** Nucleotide and amino acid trees based on alignment of *rcoT* from *T. atroviride* LU298 and *T. hamatum* LU592 (this study) and *T. reesei* (scaffold 9: 1169397 to 1172370 [http://genome.jgi-psf.org/Trire2/Trire2.home.html]). **B.** Amino acid homology tree based on alignment of *T. atroviride* LU298 *rcoT* (this study) to *rco1* orthologues from *N. crassa* (ascn#: AAB37245), *M. grisea* (ascn#: EAA46486), *G. zeae* (ascn#: XP382677), *A. nidulans* (ascn#: AAG28504) and *P. marneffei* (ascn#: AAL99251). Homology trees were constructed using DNAman™ (Lynnon Biosoft).

4.3.1.2 Analysis of the Coding Regions of *rcoT* and Orthologues

The predicted amino acid sequence of *rcoT* from *T. atroviride* was analysed using the blastp function on GenBank (http://www.ncbi.nlm.nih.gov) and aligned to the top ten most significant hits (Table 4.2). All protein sequences exhibited characteristic Rco1 (TupA) features: a conserved (57.1% identity) coiled coil domain at the N-terminus, seven highly conserved (70-94% identity) WD-repeat motifs at the C-terminus end, with a variable region (putative histone binding) in between. These features were also conserved in *T. hamatum* and *T. reesei rcoT* (Figure 4.6). The length of the coiled-coil domain varied between species, however the WD-repeat motifs were all conserved in length, except for WD7. There was also variability in the spacing between all motifs. In general the histone binding region was predominantly rich in proline and to a lesser degree glutamine and glycine, except for *Yarrowia lipolytica*, which was predominantly rich in glutamine. Sequence conservation in the histone binding region was apparent between the first six species listed in Table 4.2 (52.1% identity) and in addition two 100% identical elements (TASPGP and GPATPQ) were identified.

4.3.1.3 Genome Analysis of *rcoT* and Orthologues

Analysis of available annotated genomes revealed the presence of a conserved putative C5,6 sterol desaturase upstream of *rcoT* and its orthologues (Figure 4.7). The amino acid sequence was subjected to a blastp search on GenBank (http://www.ncbi.nlm.nih.gov), which revealed it to be an orthologue of ERG3, an enzyme involved in ergosterol biosynthesis. The distance between the translational start site sites varied from 0.6 to 7 kb and in *N. crassa* an unknown hypothetical gene lay between *rco1* and *erg3*. Protein annotation is not yet available for the *M. grisea* genome, however an *erg3* orthologue was identified using the tblastp function on GenBank (http://www.ncbi.nlm.nih.gov) and this lay 10.8 kb upstream of the *rcoT* orthologue in the opposite direction. Presence of this gene directly upstream of *rcoT* orthologues was not conserved in the basidiomycetes *U. maydis* and *C. neoformans*, or in *Saccharomyces cerevisea* and *Candida albicans*. Alignment of the sterol desaturase amino acid sequences shows they are highly conserved between species (Figure 4.8).

Table 4.2. Sequence analysis of Rco1 orthologues. Blast scores are based on blastP analysis of RcoT from *Trichoderma atroviride*.

Species	Blast score	Protein length (aa)	Coiled Coil Domain (aa position)	Putative histone binding region				WD-repeat motifs (aa position)						
				Length	aa %									
					Pro	Gln	Gly	WD-1	WD-2	WD-3	WD-4	WD-5	WD-6	WD-7
[a]*Trichoderma atroviride*		614	18-84	222	21.17	16.22	11.26	307-340	361-389	401-431	446-472	487-517	539-569	581-611
[a]*Gibberella zeae*	773	619	19-85	223	18.83	14.35	14.35	309-342	363-391	403-433	448-474	489-519	542-572	584-614
[a]*Magnaporthe grisea*	731	607	21-87	205	27.32	12.20	14.15	293-326	346-374	386-416	431-457	472-500	526-556	568-598
[a]*Neurospora crassa*	638	604	19-85	205	26.83	12.68	10.73	291-324	344-372	384-414	429-455	469-499	523-553	565-600
[a]*Aspergillus nidulans*	569	619	41-107	190	20.79	11.05	10.53	298-331	351-379	391-421	436-462	477-507	530-560	572-602
[a]*Penicillium marneffei*	569	583	15-81	190	21.58	11.05	14.74	272-305	325-353	365-395	410-436	451-481	505-535	547-577
[a]*Exophiala dermatitidis*	541	619	24-90	212	22.17	13.21	15.09	303-336	357-385	397-427	442-468	583-513	536-566	578-608
[a]*Yarrowia lipolytica*	538	647	13-86	251	13.51	29.48	7.97	338-371	391-419	431-461	476-502	517-547	569-599	611-641
[a]*Ashbya gossypii*	435	629	11-85	184	14.13	8.70	7.07	270-303	352-380	392-422	437-464	483-513	532-562	574-610
[b]*Ustilago maydis*	430	731	91-164	245	15.92	0.82	15.51	410-443	463-491	503-533	557-583	595-625	653-683	695-725
[b]*Cryptococcus neoformans*	427	564	31-104	132	17.42	3.03	7.58	237-270	290-318	330-360	386-412	424-454	484-513	526-556

[a]Ascomycotina [b]Basidiomycotina

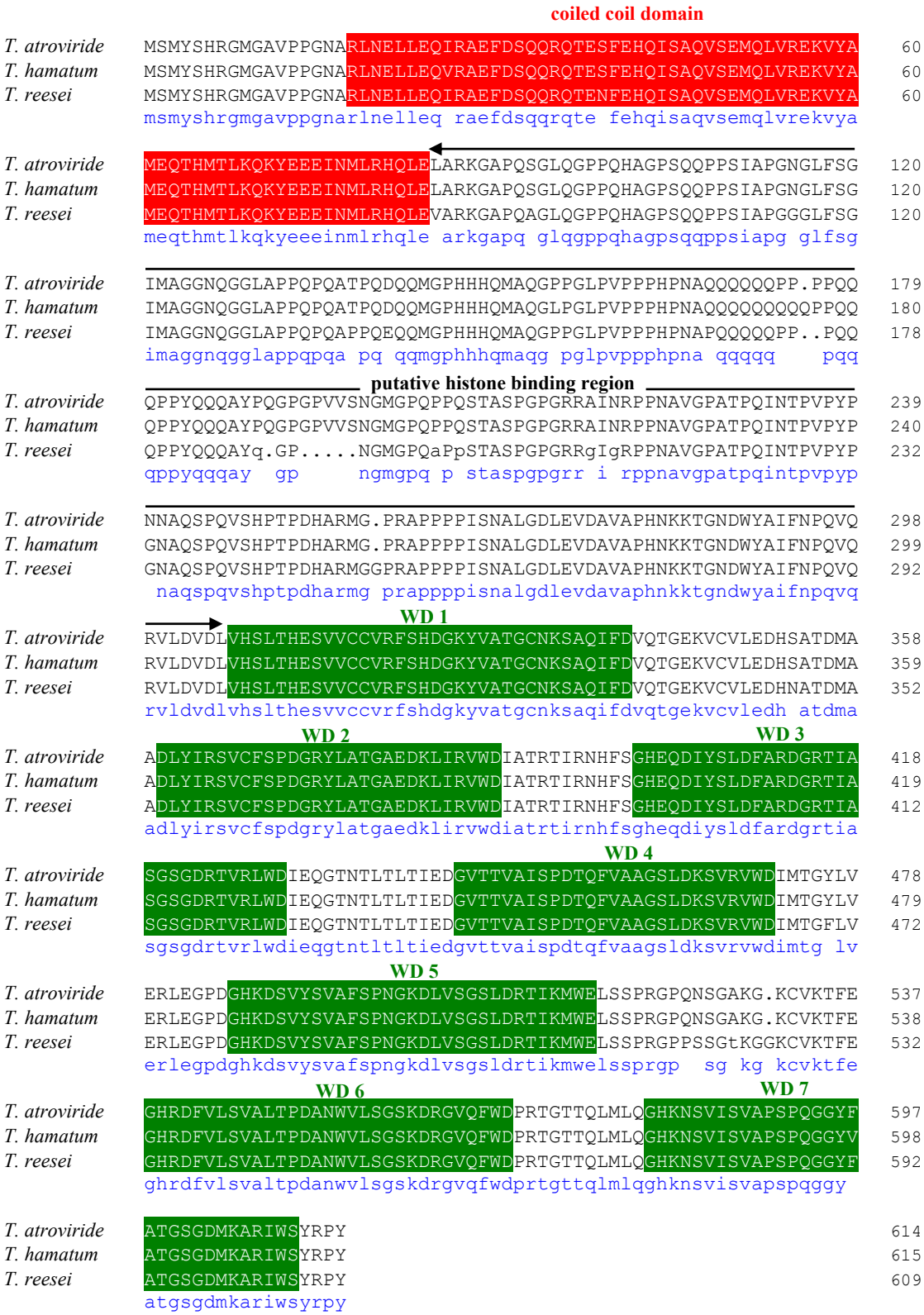


Figure 4.6. Amino acid sequence alignment of RcoT from *T. atroviride* LU298, *T. hamatum* LU592 and the *T. reesei* genome (Protein ID: 121940 [http://genome.jgi-psf.org/Trire2/Trire2.home.html]). The location of conserved domains is based on similarity to *N. crassa* Rco1, *A. nidulans* RcoA and *P. marneffei* TupA. Consensus amino acid sequences are in blue.

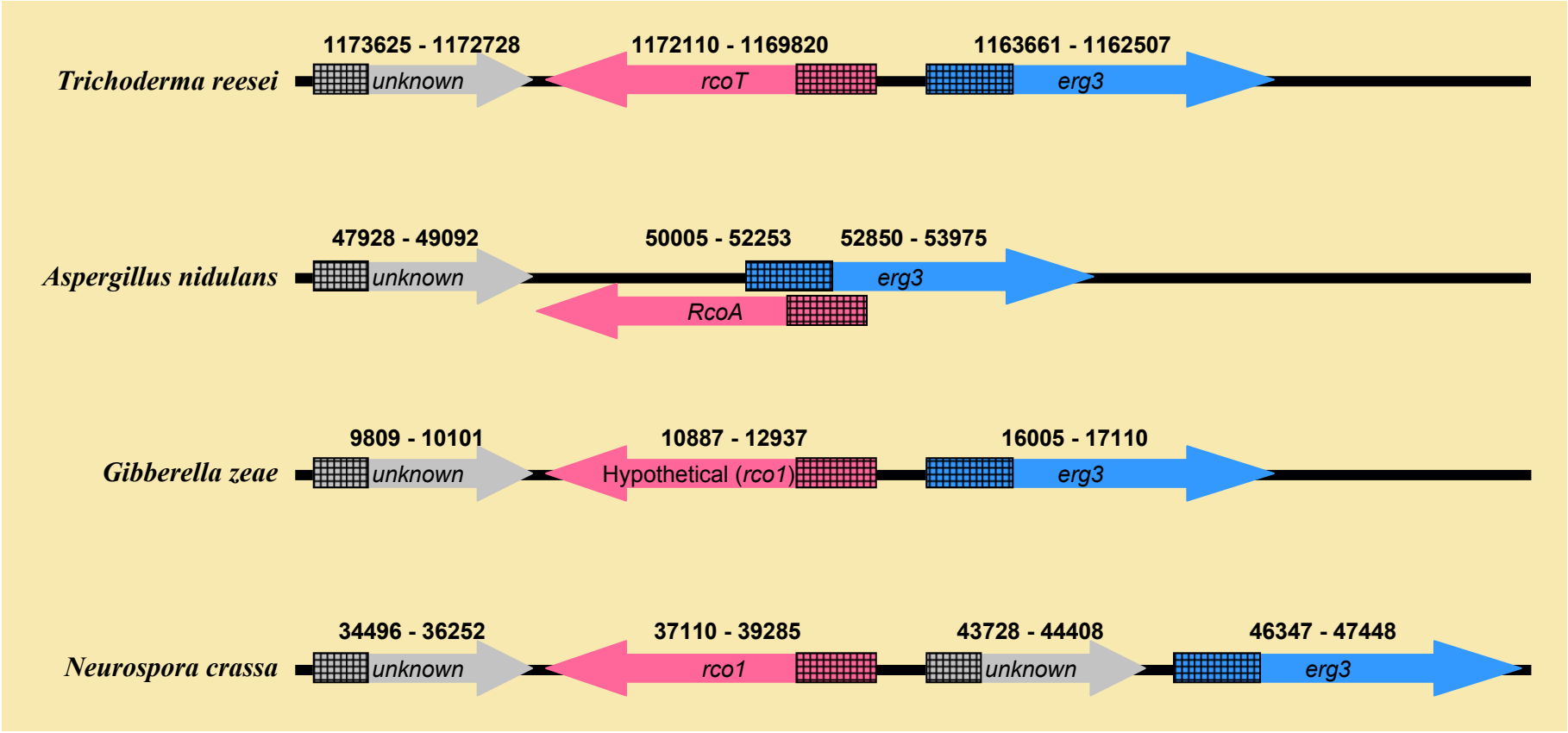


Figure 4.7. Genomic structure of *rcoT* and orthologues. Numbering refers to coding region and is relative to position within the contigs. *Trichoderma reesei* sequences were derived from the JGI website http://genome.jgi-psf.org/Trire2/Trire2.home.html (version 2.0, Scaffold 9) and all others from Genbank http://www.ncbi.nlm.nih.gov (*A. nidulans* ascn: AACD01000109.1, *G. zeae* ascn: AACM01000124, *N. crassa* ascn: AABX01000170). Shaded region represents promoter sequences.

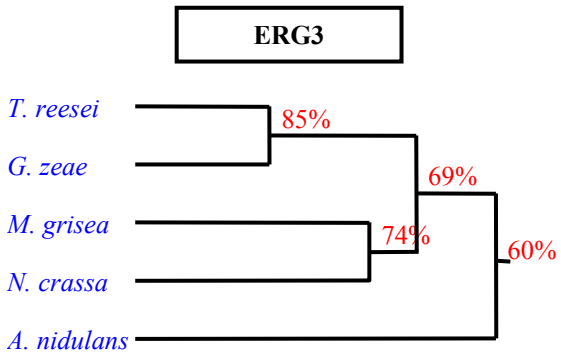
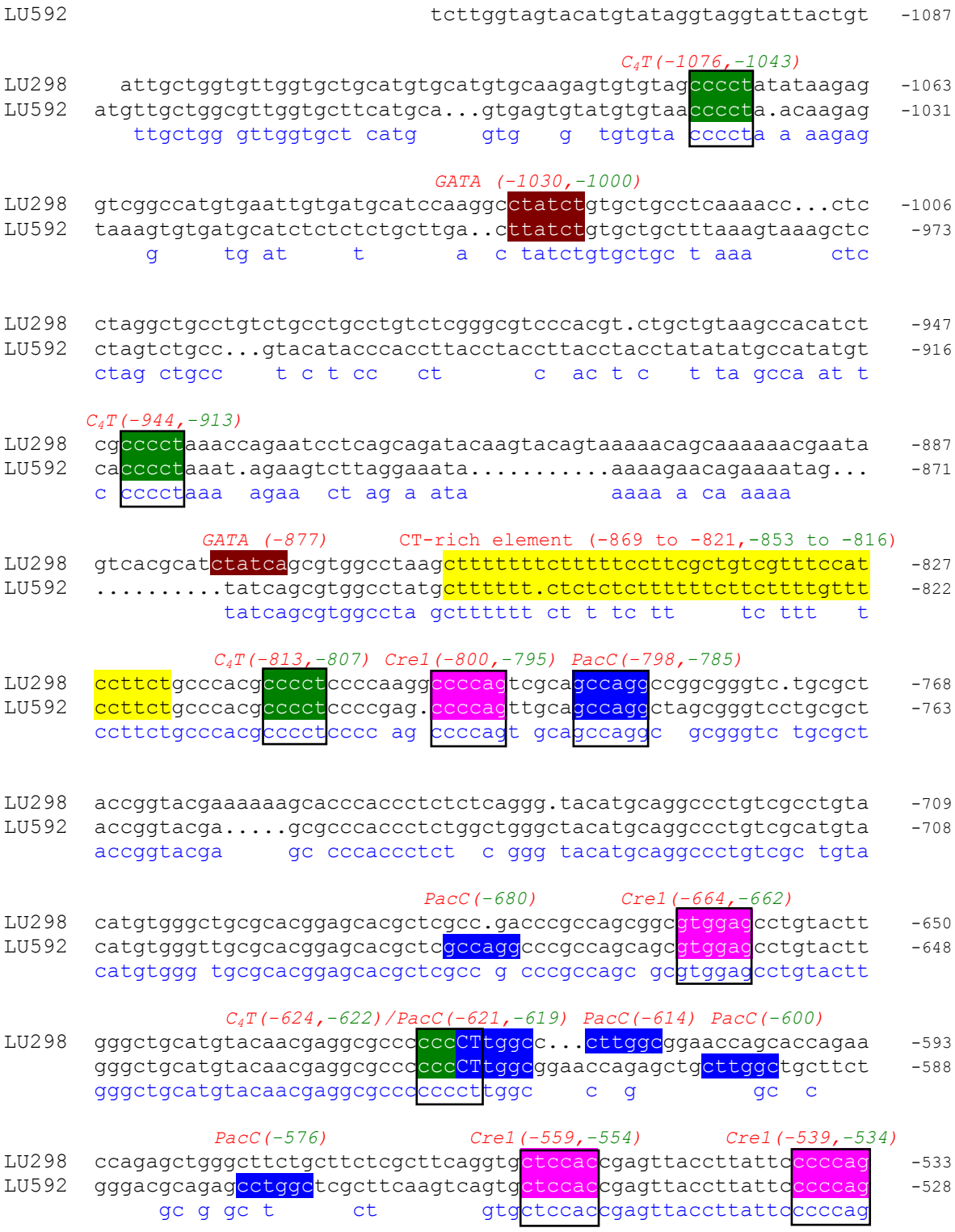


Figure 4.8. Homology tree based on amino acid alignment of ERG3 from *T. reesei* (Protein ID: 78130 [http://genome.jgi-psf.org/Trire2/Trire2.home.html]), *G. zeae* (ascn#: XP_382678), *M. grisea* (ascn#: XP_363987), *N. crassa* (ascn#: XP_962923) and *A. nidulans* (ascn#: XP_664110). Homology trees were constructed using DNAman™ (Lynnon Biosoft).

4.3.1.4 Identification of Putative Regulatory Motifs in *Trichoderma* spp.

The location of previously reported putative regulatory motifs was established through computer aided sequence searching *rcoT* from *T. atroviride* LU298 and *T. hamatum* LU592. Alignment of 1100 bp 5' to the translational start of *rcoT* revealed numerous common regulatory elements conserved between *T. atroviride* and *T. hamatum*, many of which were also conserved in *T. reesei* (Figure 4.9, Appendix 7.5). The promoter regions of *rcoT* from all three *Trichoderma* species lacked a consensus TATAA motif. A single CAAT motif was present in both *T. hamatum* (-84) and *T. reesei* (-336). Numerous Cre1 binding motifs (5'-SYGGRG-3') were present in all three species: eight in *T. atroviride*, eight in *T. hamatum* and 14 in *T. reesei*. Of these, seven were conserved between *T. atroviride* and *T. hamatum* and six between all three species. Two consensus GATA motifs (5'-HGATAR-3') were identified in *T. atroviride*, three in *T. hamatum* and one in *T. reesei*. The GATA motif at position -1030 in *T. atroviride* was conserved in *T. hamatum*, however none were conserved between all three species. Three putative PacC binding sites (5'-GCCARG-3') were identified in *T. atroviride*, four in *T. hamatum* and two in *T. reesei*. Of these, three were conserved between *T. atroviride* and *T. hamatum* and one was conserved between all three species. Four consensus C_4T motifs were conserved between all three species and both *T. hamatum* and *T. reesei* contained an additional non-conserved motif. In addition to the fungal regulatory elements identified in all three promoters three conserved CT-rich regions were present in the promoters of *T. atroviride* and *T. hamatum*. Of these two were conserved in *T. reesei*. The CT-rich regions ranged from 40 – 50 bp in length.


LU592 tcttggtagtacatgtataggtaggtattactgt -1087
C4T(-1076,-1043)
LU298 attgctggtgttggtgctgcatgtgcatgtgcaagagtgtgtagcccctatataagag -1063
LU592 atgttgctggcgttggtgcttcatgca...gtgagtgtatgtgtaacccctaacaagag -1031
ttgctgg gttggtgct catg gtg g tgtgta ccccta a aagag
GATA (-1030,-1000)
LU298 gtcggccatgtgaattgtgatgcatccaaggctatctgtgctgcctcaaaacc...ctc -1006
LU592 taaagtgtgatgcatctctctctgcttga..cttatctgtgctgctttaaagtaaagctc -973
g tg at t a c tatctgtgctgc t aaa ctc
LU298 ctaggctgcctgtctgcctgcctgtctcgggcgtcccacgt.ctgctgtaagccacatct -947
LU592 ctagtctgcc...gtacatacccaccttacctaccttacctacctatatatgccatatgt -916
ctag ctgcc t c t cc ct c ac t c t ta gcca at t
C4T(-944,-913)
LU298 cgcccctaaaccagaatcctcagcagatacaagtacagtaaaaacagcaaaaaacgaata -887
LU592 cacccctaaat.agaagtcttaggaaata..........aaaagaacagaaaatag... -871
c ccccctaaa agaa ct ag a ata aaaa a ca aaaa
GATA (-877)
CT-rich element (-869 to -821,-853 to -816)
LU298 gtcacgcatctatcagcgtggcctaagctttttttctttttccttcgctgtcgtttccat -827
LU592 ....tatcagcgtggcctatgctttttt.ctctctctttttttcttctttttgttt -822
tatcagcgtggccta gctttttt ct t tc tt tc ttt t
C4T(-813,-807)
Cre1(-800,-795)
PacC(-798,-785)
LU298 ccttctgcccacgcccctccccaaggccccagtcgcagccaggccggcgggtc.tgcgct -768
LU592 ccttctgcccacgcccctccccgag.ccccagttgcagccaggctagcgggtcctgcgct -763
ccttctgcccacgcccctcccc ag ccccagt gcagccaggc gcgggtc tgcgct
LU298 accggtacgaaaaaagcacccaccctctctcaggg.tacatgcaggccctgtcgcctgta -709
LU592 accggtacga.....gcgcccaccctctggctgggctacatgcaggccctgtcgcatgta -708
accggtacga gc cccaccctct c ggg tacatgcaggccctgtcgc tgta
PacC(-680)
Cre1(-664,-662)
LU298 catgtgggctgcgcacggagcacgctcgcc.gacccgccagcggcgtggagcctgtactt -650
LU592 catgtgggttgcgcacggagcacgctcgccaggcccgccagcagcgtggagcctgtactt -648
catgtggg tgcgcacggagcacgctcgcc g cccgccagc gcgtggagcctgtactt
C4T(-624,-622)/PacC(-621,-619)
PacC(-614)
PacC(-600)
LU298 gggctgcatgtacaacgaggcgcccccccCTtggcc...cttggcggaaccagcaccagaa -593
gggctgcatgtacaacgaggcgcccccccCTtggcggaaccagagctgcttggctgcttct -588
gggctgcatgtacaacgaggcgcccccctggc c g gc c
PacC(-576)
Cre1(-559,-554)
Cre1(-539,-534)
LU298 ccagagctgggcttctgcttctcgcttcaggtgctccaccgagttaccttattccccccag -533
LU592 gggacgcagaggcctggctcgcttcaagtcagtgctccaccgagttaccttattccccccag -528
gc g gc t ct gtgctccaccgagttaccttattccccccag

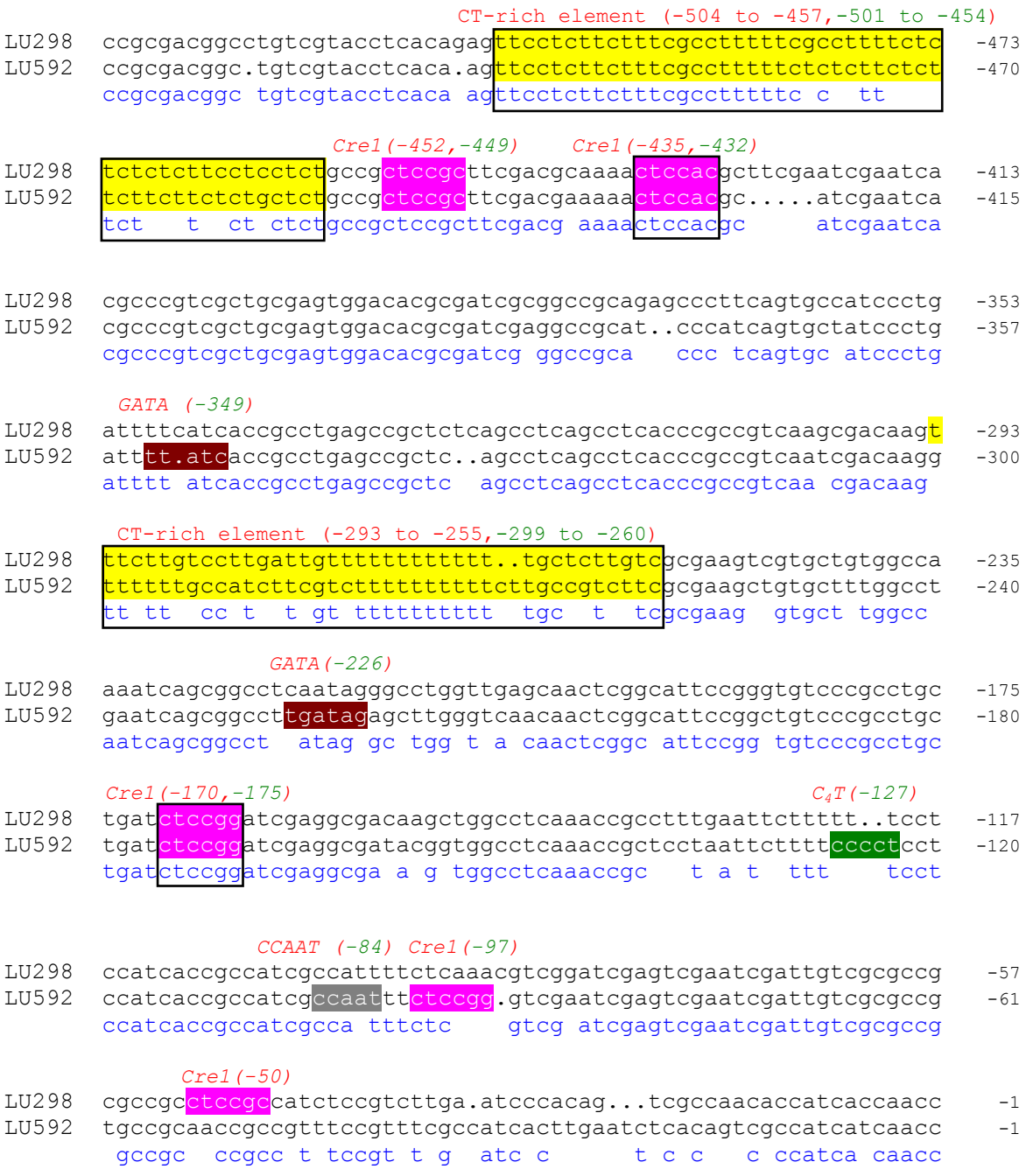


Figure 4.9. Alignment of *rcoT* from *T. atroviride* LU298 and *T. hamatum* LU592 upstream of the ATG start codon. Numbering is relative to the transcriptional start site at +1. CT-rich elements are shaded in yellow. Where two motifs overlap, bases are in upper case. Consensus sequences are in blue.

4.3.1.5 Comparison of the *rcoT* Gene Orthologue Regulatory Regions

Binding motifs common to the promoters of *rcoT*, *rco1* and *TupA* may suggest commonalities in gene regulation between orthologous genes. Using the genomic libraries present on GenBank (http://www.ncbi.nlm.nih.gov) 1100 bp of sequence 5' to the *rco1*(*TupA*) translational start sites were obtained from *G. zeae*, *M. grisea*, *N. crassa* and *A. nidulans* and analysed as described above (Figure 4.10, Appendix 7.5). No consensus TATAA motif was identified in all promoters examined, suggesting *rcoT* (*rco1*, *TupA*) to have a TATA-less promoter. Numerous Cre1 and C_4T binding motifs were identified in all promoters, suggesting carbon source and stress may be common regulatory factors. GATA and PacC motifs were identified in all promoters except *N. crassa* and CT-rich elements in all except *M. grisea*.

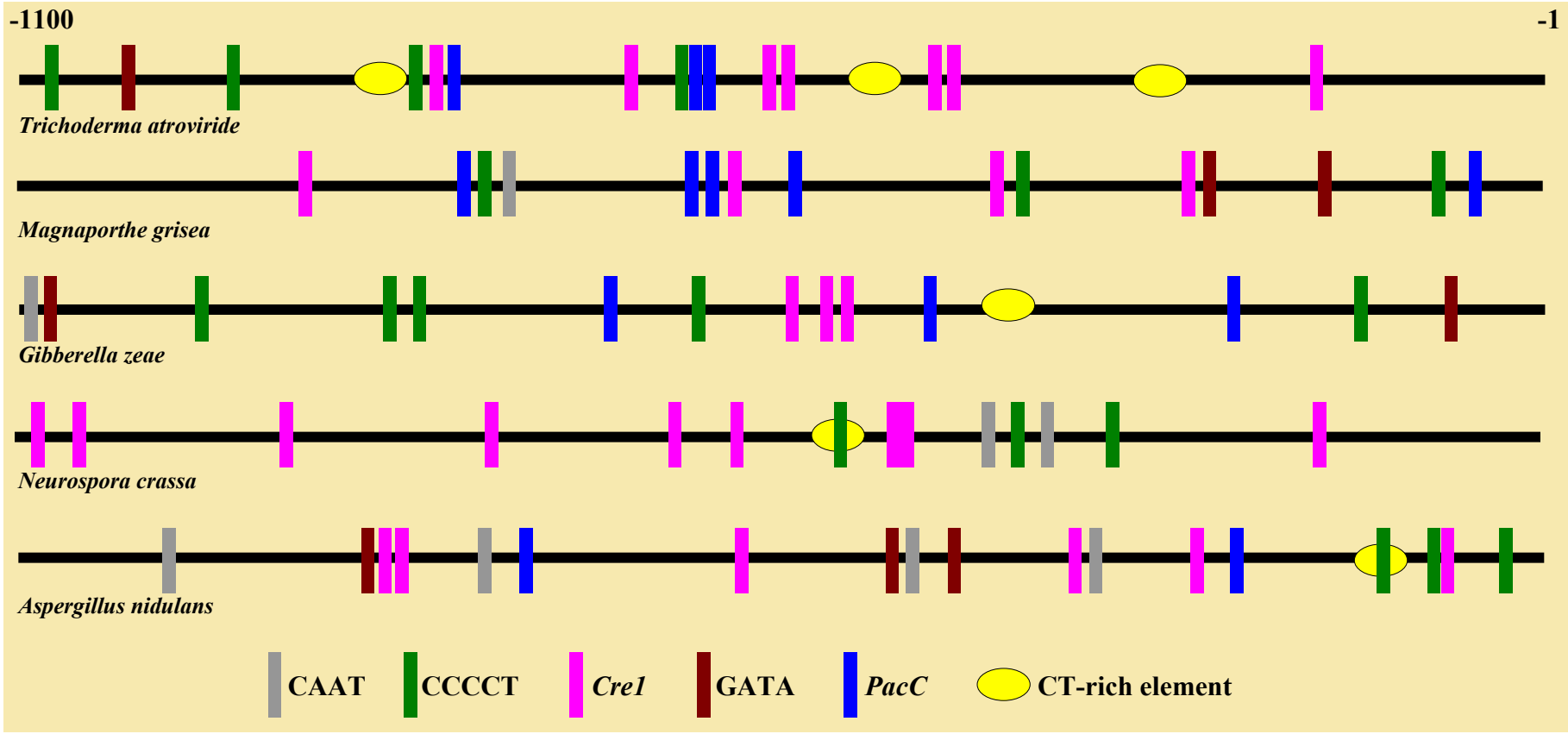


Figure 4.10. Alignment of the first 1100 bp 5' to the transcriptional start point (TSP) in *rcoT* and orthologues. Motifs that are in line with each other are equidistant to the TSP within 50 bp.

4.3.2 Isolation of *con-10* Gene Orthologues from *Trichoderma* spp.

Using a combination of genome mining and degenerate PCR, putative orthologues of the *con-10* gene were identified from *T. reesei*, *T. atroviride* and *T. hamatum*. Blast analysis of the *T. reesei* genome identified an annotated putative *con-10* sequence with approximately 71% amino acid identity to *N. crassa con-10*. On the basis of alignment between *T. reesei* and *N. crassa* Con-10, degenerate primers were designed and successfully used to isolate a portion of the orthologous gene from *T. atroviride* and *T. hamatum*. Using primers Con10-5pF and Con10-62pR a single band of 262 and 266 bp was amplified and sequenced from *T. atroviride* and *T. hamatum*, respectively. Predicted sequences of 50 amino acids for *T. atroviride* and *T. hamatum* Con-10 were derived using the Wise2 program (http://www.ebi.ac.uk/Wise2). Alignment of the *Trichoderma* spp. and *N. crassa con-10* sequences revealed a high degree of conservation at both the nucleotide and amino acid level, however, this was considerably less than that observed between *rcoT* and *rco1* orthologues (Figure 4.11). The partial sequence isolated from *T. atroviride* and *T. hamatum* encompassed both introns, which proved problematic for nucleotide sequence alignment, therefore the nucleotide homology tree is based on the predicted cDNA sequence derived using the Wise2 program. Sequence alignments of *con-10* from *T. atroviride* and *T. hamatum* are presented in Appendix 7.4.4.

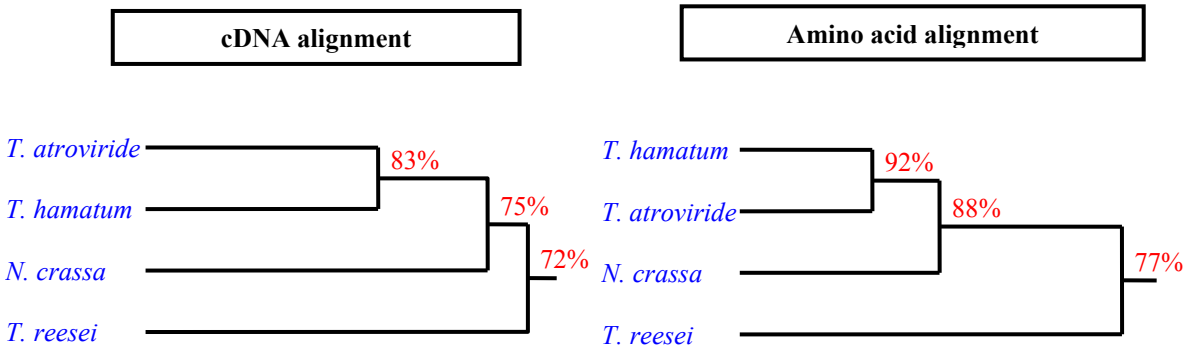


Figure 4.11. Homology trees based on alignment of predicted mature mRNA from *con-1* orthologues from *T. atroviride* LU298 and *T. hamatum* LU592 (this study), *T. reesei* (Scaffold 19: 258000-259000 [http://genome.jgi-psf.org/Trire2/Trire2.home.html]) and *N. crassa* (ascn#: M20005). Homology trees were constructed using DNAman™ (Lynnon Biosoft).

In *N. crassa con-10*, promoter analysis has identified an element associated with mycelial repression, which has been implicated to be involved in con-10 response to *rco-1* activity (Corrochano *et al.*, 1995; Yamashiro *et al.*, 1996). Analysis of the promoter sequence of the putative *con-10* gene from *T. reesei* revealed one conserved 5'-GGGAGCT-3' motif at -144 bp, relative to the ATG start codon at +1.

4.3.3 Generation of *Trichoderma* spp. *rcoT* Mutants

4.3.3.1 *rcoT* Gene Replacement Experiments

Using Agrobacterium-mediated transformation, attempts were made to create *T. atroviride* LU298 and *T. hamatum* LU592 knock-out strains in which the *rcoT* coding region was replaced by *hph* (hygromycin phosphotransferase) from *E. coli* via homologous recombination. Background sensitivity to Hygromycin-B was determined using PDA amended with increasing levels of the antibiotic (Figure 4.12). At 200 µg/mL Hygromycin-B, no growth was detected in both isolates and 300µg/mL was chosen as the selection concentration for the gene replacement transformations.

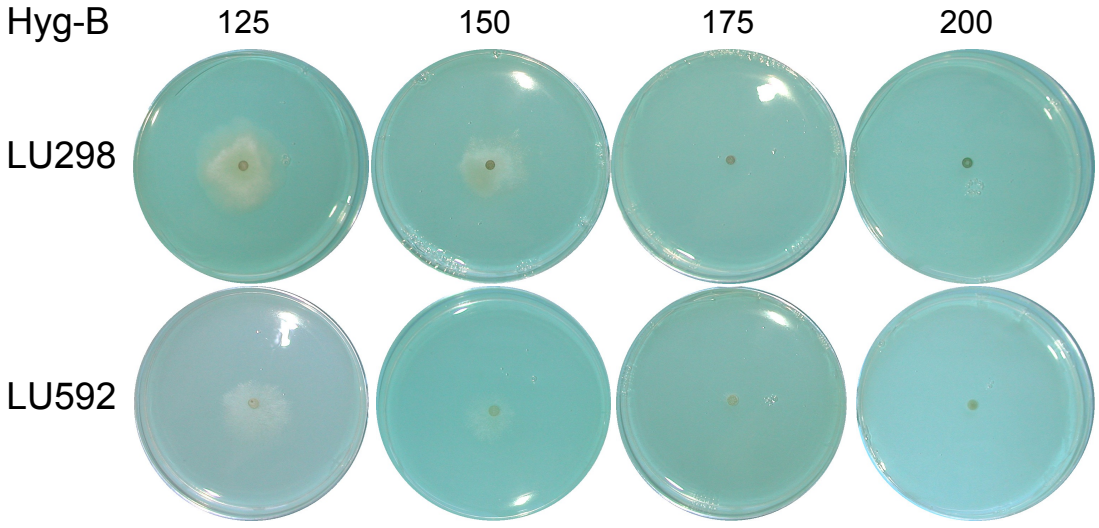


Figure 4.12. Growth of *T. atroviride* LU298 and *T. hamatum* LU592 on PDA amended with 125-200 µg/mL Hygromycin-B.

Multiple rounds of transformation were done on both isolates with the gene replacement vectors however no homologous recombinant was obtained. The transformation rate of *T. atroviride* LU298 was one in 3 X 10^6 and *T. hamatum* LU592 was approximately double that. In the first experiment three Hygromycin B resistant colonies were recovered from LU592 (Rx592A1-A3) and one from LU298 (Rx298A1). PCR analysis was initially done 2-3 days after removal from the overlay plates to PDA with 300

µg/mL Hygromycin B using mycelium scraped from the actively growing margin. Putative transformants were tested for homologous integration using primers designed to a region upstream of the predicted T-DNA integration point and within the *hph* gene on the T-DNA construct (lane A Figure 4.13). As a negative control, primers were also designed to amplify the portion of *rcoT* predicted to be replaced (lane B, Figure 4.13). In addition, attempts were made to amplify the *hph* gene using primers hpha and hphb as a test for T-DNA transformation (Lane C, Figure 4.13). No homologous integration was detected in any strain and only two of the *T. hamatum* Hygromycin B resistant strains (Rx592A1 & A3) were positive for *hph*, however amplification was low (Figure 4.13).

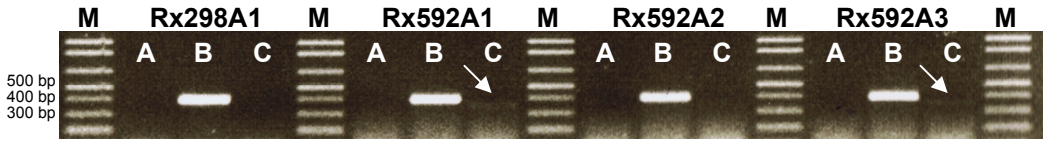


Figure 4.13. PCR analysis of putative *rcoT* gene replacement transformants. **A.** Primers were designed to test for homologous recombination. **B.** Amplification of the wild-type *rcoT*. **C.** Amplification of the *hph* gene. M = Molecular weight marker. Arrow indicates weak amplimer.

Poor amplification of *hph* in comparison to the *rcoT* amplimer suggested a small portion of nuclei were transformed in comparison to the total tissue used for DNA extraction. Therefore it is possible strains which gave negative PCR results for *hph* and the homologous integration boundary may contain copies of both intact *rcoT* and *rcoT* knock-out loci at a level below PCR detection. However, it is also possible that the T-DNA integrated outside the target locus by non-homologous recombination and that there were no *rcoT* knock-outs. It is also possible, but unlikely, that there was still some *Agrobacterium* present and binary vector within Agrobacterium was detected. A nested PCR strategy to detect the knock-out loci was adopted to increase detection sensitivity. All DNA samples from the first experiment were re-tested and in the secondary PCR strong amplification of the *hph* gene was observed from all four *Trichoderma* spp. strains (Figure 4.14). No homologous integration was detected using nested PCR, suggesting that no rcoT knock-outs were achieved.

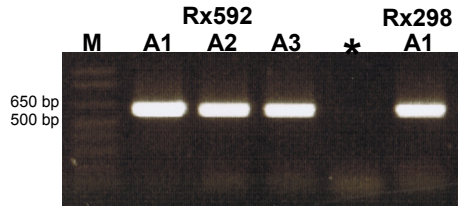


Figure 4.14. Nested PCR detection of *hph* from *T. hamatum* and *T. atroviride* transformants created in the *rcoT* gene replacement experiments. M = Molecular weight marker. * = Nested amplification of the negative control from the primary PCR reaction.

Three further gene replacement transformation experiments were conducted on *T. atroviride* LU298 and two on *T. hamatum* LU592 and all putative transformants were PCR analysed using the nested PCR strategy. In total 21 *T. atroviride* LU298 and 41 *T. hamatum* LU592 Hygromcyin B resistant colonies were obtained from all experiments. Of these, *hph* was amplified in the primary PCR from two LU298 strains and 20 LU592 strains. In the secondary PCR *hph* amplification was positive for six additional LU298 strains and 14 additional LU592 strains. One strain only, from *T. hamatum* (Rx592C47), produced a band indicating homologous integration in the secondary PCR (Figure 4.15), though *hph* was strongly amplified in the primary PCR suggesting both ectopic and homologous integration of the gene replacement construct. Rx592C47 was sub-cultured to eight fresh selection plates (a-h) and incubated further. DNA was prepared from all eight plates and subjected to nested-PCR analysis (Figure 4.15). No homologous integration band was detected in any of the samples and *hph* was detected in the secondary PCR only from five out of the eight subcultures. In each case the *rcoT* gene was amplified strongly using primary PCR only.

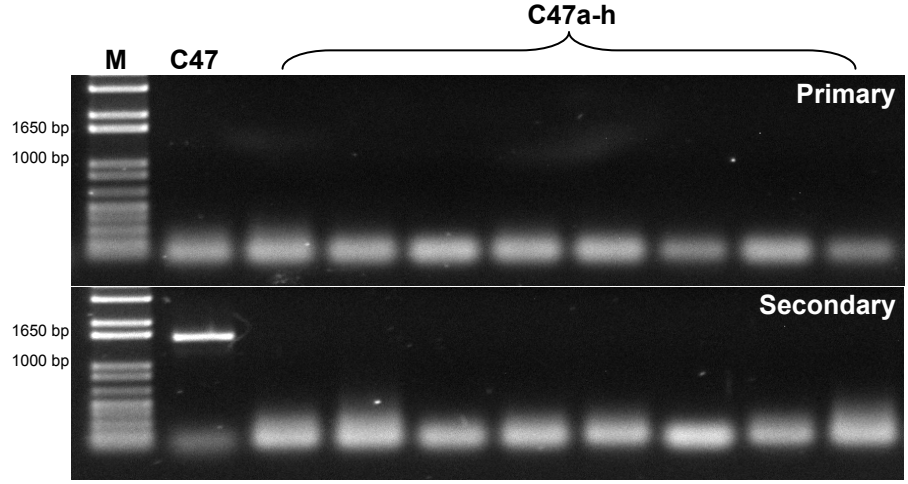


Figure 4.15. Nested PCR detection of homologous recombination from *T. hamatum* C47. Nested PCR was done on gDNA from 5 old colonies (C47) and from 8 first round sub-cultures (C47a-h), which were also 5 days old. M = Molecular weight marker.

The low level of *hph* copies in some Hygromycin B resistant strains suggested untransformed tissue was growing on the selection plates and in the case of Rx592C47 the proportion of untransformed nuclei increased relative to transformed nuclei. In Chapter Two it was demonstrated that *Trichoderma* spp. modify the ambient pH of unbuffered media. If Hygromycin B efficacy is influenced by ambient pH, then lowering the pH of the selection plates may allow untransformed tissue to grow in the presence of selection. To test whether ambient pH affects background sensitivity to selection, *T. atroviride* was inoculated to PDA which has been acidified to pH 4.0 and amended with 125 to 475 µg/mL Hygromycin B at 25 µg intervals. Spores were able to germinate at up to 425 µg/mL Hygromycin B (Figure 4.16) demonstrating that low ambient pH does reduce Hygromycin B effacacy. Given that *Trichoderma* actively acidify the medium it was decided to use PDA acidified to pH 4.0 and amended with 600 µg/mL for all further transformation experiments.

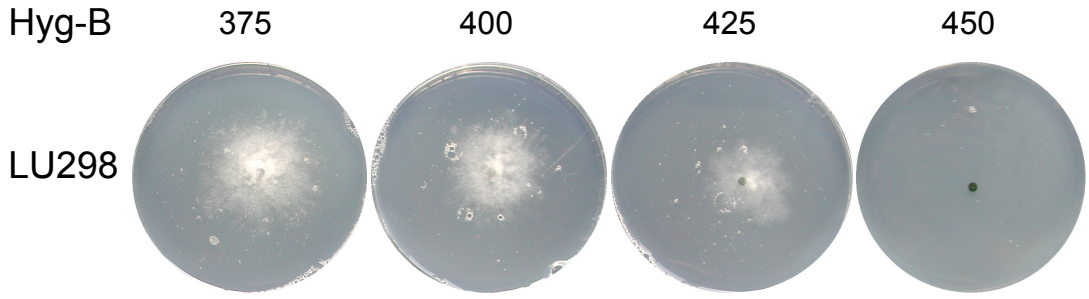


Figure 4.16. Growth of *T. atroviride* LU298 and *T. hamatum* LU592 on PDA, acidified to pH 4.0 and amended with 375-450 µg/mL Hygromycin-B.

4.3.3.2 Creation of *T. atroviride* Antisense Transformants

The rate of transformation was very low using the gene replacement vectors and whilst ectopic integration could be achieved only one out of 62 resistant strains showed evidence of homologous integration, though this was not stable. No further attempts were made at creating gene replacement strains and instead an antisense strategy was used to create *T. atroviride rcoT* knockdown mutants. A total of 12 *T. atroviride* Hygromycin B resistant colonies (RAS1 to RAS12) were recovered from one transformation experiment using the *rcoT* antisense vector pRAS and a selection level of 600 µg/mL. All except RAS9 were PCR positive for *hph* using primers hpha and hphb only. The transformation rate was one in 8 X 10^7, which was lower than that for the gene replacement vectors. Of the remaining 11 strains, all except RAS3 continued to grow in culture and were able to be single-spore purified. PCR analysis was repeated on tissue from the purified strains to confirm the presence of *hph* (Figure 4.17).

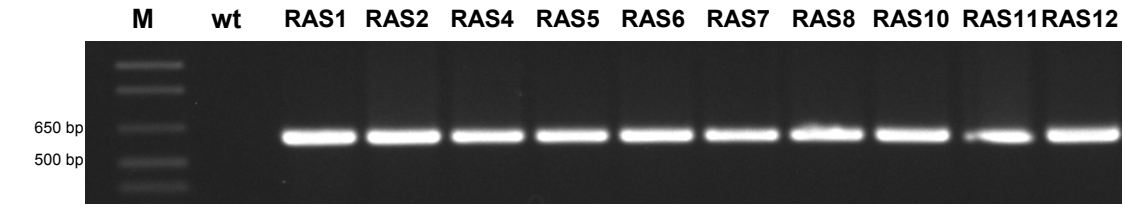


Figure 4.17. PCR amplification of the *hph* gene from *T. atroviride* LU298 and all antisense transformants. M = Molecular weight marker.

4.3.3.3 Southern Analysis of Antisense Strains

To confirm genomic integration of the T-DNA construct, Southern analysis of the presence of *hph* was done on genomic DNA from *T. atroviride* LU298 and all 10 *rcoT* antisense strains so that the number of independent integration loci can be assessed. Presence of the *hph* gene was detected in the antisense strains only (Figure 4.18). Integration appeared to be single copy in all strains except RAS6, in which two bands were detected.

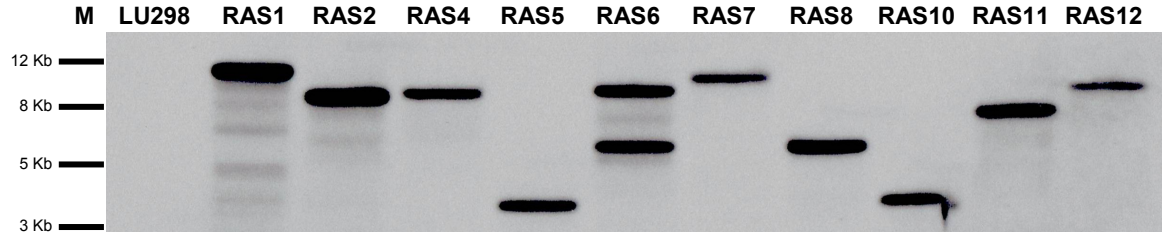


Figure 4.18. Southern Analysis presence of *T. atroviride* LU298 and *rcoT* antisense derivatives. Southern blot was hybridised to a chemiluminescent-labelled *hph* DNA probe.

4.3.4 Gene Expression Analysis of Antisense Strains

4.3.4.1 Dot Blot Analysis of *rcoT* Expression

To determine if integration of the *rcoT* antisense vector resulted in a reduction in the *rcoT* transcript level, RNA dot-blot analysis was done on *rcoT* from *T. atroviride* LU298 and all antisense mutants. *rcoT* expression was investigated using three dot blots, to which RNA from dark grown cultures had been applied. The first blot was hybridised to a DNA probe, which detects both sense and antisense RNA. Strong expression of *rcoT* was observed in the wild-type and, unexpectedly, in all antisense mutants (Figure 4.19A). To determine whether the *rcoT* mRNA detected in the antisense strains derived from the wild-type gene or from the integrated antisense construct, replica blots were probed with either a sense- or antisense-specific RNA probe. Strong expression of the wild-type *rcoT* gene was observed in the wild-type and

all antisense transformants, suggesting no reduction in *rcoT* transcript levels (Figure 4.19B). In contrast, expression of the antisense version of *rcoT* appeared strong in RAS5, RAS6 and RAS11, moderate in RAS1 and RAS8 and weakly expressed in all other transformants (Figure 4.19C). No antisense *rcoT* should be present in wild-type RNA sample, however a weak signal was observed from the wild-type LU298 on blot 3.

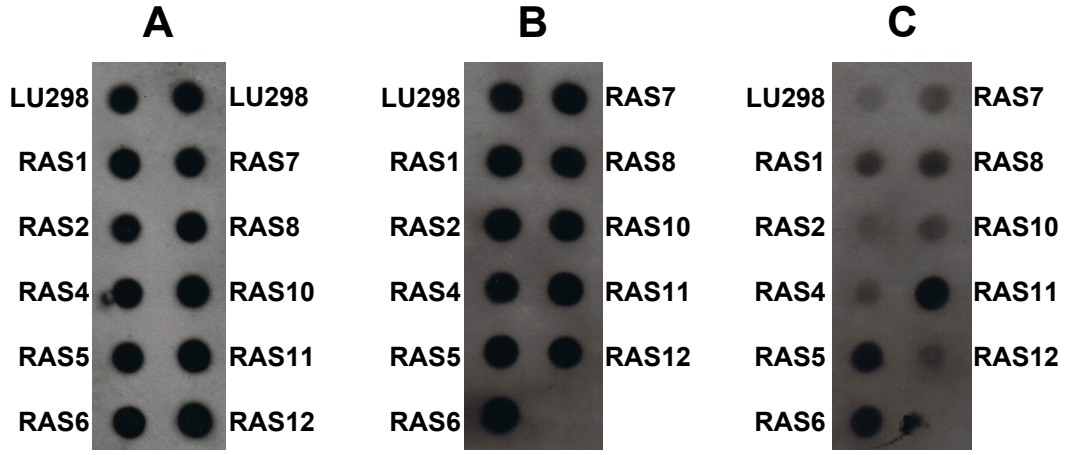


Figure 4.19. RNA dot-blot analysis of *rcoT* from *T. atroviride* and *rcoT* antisense mutants created in this study. **A.** Blot 1: 20 µg total RNA was loaded into each well and the blot was hybridised to an *rcoT* DNA probe. **B.** Blot 2: 10 µg total RNA was loaded into each well and the blot was hybridised to an *rcoT* RNA probe specific to sense (wild-type) *rcoT*. **C.** Blot 3: 10 µg total RNA was loaded into each well and the blot was hybridised to an *rcoT* RNA probe specific to antisense *rcoT*.

4.3.4.2 Northern Analysis of *rcoT* and *con-10* Expression

To determine whether *con-10* expression was up-regulated in the antisense transformants, northern analysis was done on *con-10* from *T. atroviride* LU298 and all antisense mutants. No *con-10* expression was detected in any sample (Figure 4.20). The northern blot was also probed with the *rcoT* DNA probe used in 4.3.4.1 and as observed on the dot-blots, expression was high in all samples.

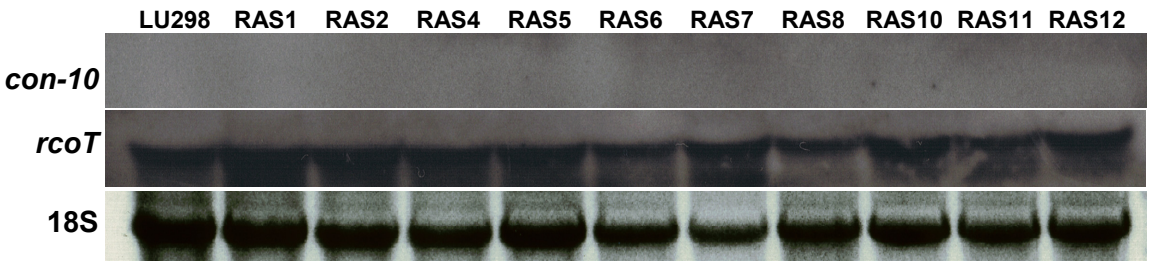


Figure 4.20. Northern analysis of *con-10* and *rcoT* expression in *T. atroviride* LU298 and *rcoT* antisense mutants created in this study. Approximately 15 µg of total RNA was loaded into each lane, and the blot was hybridised to *con-10* or *rcoT* DNA probes. Ethidium bromide-stained 18S rRNA is shown as a loading control.

4.3.5 Morphological Analysis of Antisense Strains

Mycelial growth rates and the effect of alternating light/dark exposure on conidiation was investigated in *T. atroviride* LU298 and all antisense transformants. RAS10 was the only transformant which differed significantly from the wild-type. Growth rates ranged from 1.8 to 2.0 mm per hour for the wild-type and all transformants, except RAS10, which grew at 0.8 mm per hour. Similarly, all transformants, except RAS10, resembled the wild-type cultures when incubated under alternating light/dark conditions. Typical rings of conidia were observed on the LU298 plates, whereas the rings were tightly compacted on the RAS10 plates (Figure 4.21). In addition, the rings on the RAS10 were slightly irregular in shape.

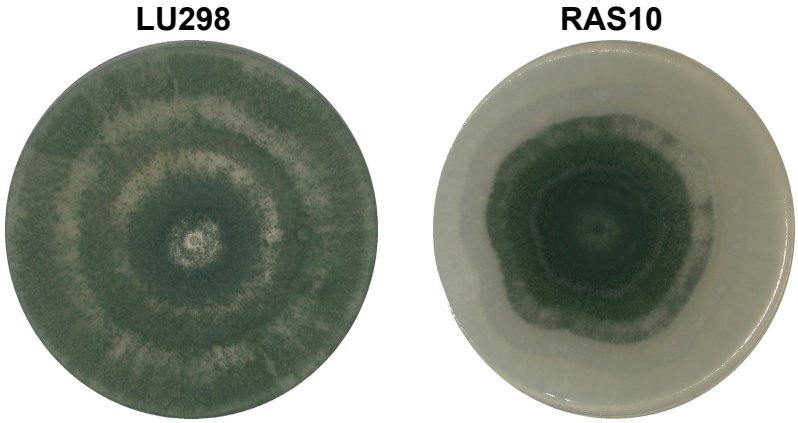


Figure 4.21. Effect of alternating light/dark conditions on conidition in LU298 and RAS10.

4.4 Discussion

4.4.1 RcoT is a Sequence Orthologue of the Global Regulator Tup1

The predicted amino acid sequences of *T. atroviride* and *T. hamatum rcoT* are similar to that of *N. crassa rco-1*, *A. nidulans rcoA* and *P. marneffei tupA*, which have been proposed as orthologues of *tup1* from *S. cerevisiae* and *C. albicans*. All 7 WD-repeat domains are present in RcoT, and, as observed in Rco-1, RcoA and TupA, each domain was more conserved with the same domain in other species than compared to other domains within the same sequence (Yamashiro *et al.*, 1996; Hicks *et al.*, 2001; Todd *et al.*, 2003). A conserved coiled coil domain was also present at the N-terminus of RcoT and the intervening region between the coiled coil domain and the first WD-40 repeat was rich in proline, which has been suggested in other orthologues to replace the function of the glutamine- and alanine-rich region in Tup1. Indeed, a survey of the ten

closest blastp hits from RcoT revealed the proline-rich region to be common to all except the dimorphic yeast *Yarrowia lipolytica*. Based on the sequence similarity, it is proposed that the *rcoT* gene isolated in this study from *T. atroviride* and *T. hamatum* is an orthologue of *rco-1*, *rcoA*, *tupA* and *tup1*.

Using the publicly available annotated genomes, a search for *tup1* orthologues revealed the conservation in location and sequence of a gene involved in ergosterol biosynthesis. In every ascomycetous genome assessed, an *erg3* orthologue lay downstream and in the opposite direction to the *rcoT* orthologues and in *A. nidulans*, the promoter regions overlapped each other. Ergosterol is the major sterol component of fungal cellular membranes; the equivalent sterol in mammalian cells is cholesterol (Parks & Casey, 1995). In *S. cerevisiae*, loss of ergosterol biosynthesis results in mating defects (Tomeo *et al.*, 1992). Recently it has been shown in *S. cerevisiae* that Tup1 negatively regulates *erg* genes through binding to the Hap1 transcription factor (Hickman & Winston, 2007). In *Aspergillus fumigatus* the loss of the orthologous *erg3* gene results in a dramatic reduction in sterol biosynthesis (Alcazar-Fuoli *et al.*, 2006). The *A. nidulans rcoA* knockout constructs were created using 1.4 kb of sequence 5' to the *rcoA* coding region (Hicks *et al.*, 2001), which would have contained about 850 of the 1125 bp coding region of the *erg3* gene. It is possible that the Δ*rcoA* phenotype was due, in part, to *erg3* gene disruption.

Inter-species conservation of two genes in position is seldom seen except in biosynthetic pathways, where often the regulatory and structural genes cluster together, such as in aflatoxin biosynthesis (Bok & Keller, 2004), however Tup1 has been shown to be a global repressor (Smith & Johnson, 2000), therefore it would seem unlikely to be involved in a pathway-specific gene cluster. Although a regulatory relationship has been shown to exist between *tup1* and *erg3* (Hickman & Winston, 2007), unlike other biosynthetic pathways, there is no evidence to suggest that the *erg* genes and their regulators cluster together. Indeed in *S. cerevisiae*, genomic analysis showed no proximity between *tup1* and *erg3* (this study). Whether the localisation of *erg3* downstream from the ascomycetous *tup1* orthologues has a functional basis, or, is purely coincidental, is not known. It is tempting to speculate however; that the co-localisation of these genes may represent part of an ancient gene cluster conserved within the ascomycetes only, and as such, could be developed as a systematic feature for imperfect or sterile ascomycetes.

4.4.2 Multiple Elements are Conserved in the *rcoT* (*rco-1* & *rcoA*) Promoters

Sequence analysis of the promoter region of *rcoT* and other *tup1* orthologues from filamentous fungi revealed the presence of multiple regulatory elements, however only Cre1 (CreA) and C_4T binding sites were found in all promoters. Both carbon starvation and stress have been implicated in Tup1 (Rco-1) function, which suggests a possible role for the conserved promoter binding elements. In the presence of sufficient glucose, the *T. reesei* Cre1 protein binds to the promoters of genes repressing their expression, which is termed Carbon Catabolite Repression (CCR) (Cziferszky *et al.*, 2002). The functional equivalent of the Cre1 protein in the yeasts is Mig1, which accomplishes negative regulation through the recruitment of the Tup1-Ssn6 complex (Williams *et al.*, 1991; Treitel & Carlson, 1995). Despite the similarities between Cre1 and Mig1, Rco-1 (RcoA) appears to play a weak role only in carbon catabolite repression in *N. crassa* and *A. nidulans*. In a *N. crassa* Δ*rco-1* background, hyphal expression of the conidia-specific gene, *con-10*, is greatly elevated in the presence of high amounts of glycerol or in the absence of a carbon source (Lee & Ebbole, 1998). These authors suggest that Rco-1 plays a role in the prevention of mycelial expression of *con-10* in response to carbon derepressing conditions. In *A. nidulans*, loss of the *cre1* orthologue, *creA*, had no effect on *rcoA* expression and conversely, loss of *rcoA* had no effect on carbon repression of α-amylase expression, however a minimal effect was observed on alcohol dehydrogenase expression, which suggested a weak role in CCR (Hicks *et al*., 2001).

Multiple copies of the stress response element CCCCT were also observed in all *rcoT* orthologues promoters. In *S. cerevisiae*, stress response is mediated by binding of Msn2p/Msn4p to a C_4T element (Treger *et al.*, 1998). The msn2/msn4 orthologue in *T. atroviride*, *seb1*, has been demonstrated to regulate genes in response to osmotic stress (Peterbauer *et al.*, 2002). In *N. crassa*, expression of *con-10* was elevated in response to heat shock stress in a Δ*rco-1* background, which suggested that, like carbon starvation, Rco-1 is involved in repressing the stress response in mycelial tissue.

The presence of consensus binding elements within gene promoters is not, in itself, evidence for transcriptional regulation (Nussinov, 1990). A comprehensive expressional analysis of the filamentous *tup1* orthologue has not been undertaken, however from the studies to date it would appear that expression of *tup1* is constant, which is in accordance with the role of Tup1 as a global regulator. Expressional analysis of *rcoT* in

response to a range of carbon sources and stresses is required to test for transcriptional regulation.

4.4.3 Creation of an *rcoT* Mutant

A verifiable *rcoT* loss-of-function mutant was not obtained in our mutagenesis experiments. One putative *rcoT* gene replacement recombinant was isolated from *T. hamatum*, but only a portion of the nuclei were transformed and a pure culture was not obtained. This was not unexpected as mutants in both *A. nidulans* and *P. marneffei* had severe growth defects and were difficult to maintain (Nancy Keller, Richard Todd, pers comm.). Indeed the deleterious effect of loss of this gene was likely a contributing factor to the lack of success in creating knockout mutants.

Multiple antisense transformants were obtained, however RNA analysis alone was not sufficient to determine if there was any effect on production of RcoT. No reduction in the native transcript level was observed in the dot-blot RNA analysis and it was not clear if the antisense construct was being expressed in all transformants. In *N. crassa*, expression of the conidiation gene *con-10*, is conidial specific in the wild-type and aberrantly expressed in hyphal tissue of *rco-1* loss-of-function mutants (Yamashiro *et al.*, 1996). No *con-10* expression was observed in mycelial tissue of the transformants, however it is not known if *con-10* follows a similar pattern of expression and regulation in *T. atroviride*, therefore this cannot be used as a marker of loss of RcoT. Given the difficulty observed in obtaining gene knockout mutants, it is possible that only antisense transformants in which RcoT production was minimally affected or not effected at all were able to survive the transformation procedure. Protein analysis is required to determine whether incorporation of the antisense construct affected RcoT levels.

Due to the uncertainty over the exact nature of the antisense transformants, preliminary analysis only, of the morphological phenotypes, was undertaken. Of the ten transformants, RAS10 was the only isolate to be significantly altered from wild-type in growth rate and morphology. RAS10 exhibited a growth rate less than half that of the wild-type and, likely as a result of the growth rate, tightly compacted rings of conidia were produced under an alternating light/dark regime. In addition, the colony margins were irregular. Reduced growth rate and irregular colony growth were also observed in *N. crassa rco-1*, *A. nidulans rcoA* and *P. marneffei tupA* loss of function mutants.

Chapter Five
Implications for *Trichoderma* Research

One third of all fungal biocontrol agents (BCAs) are isolates of *Trichoderma*, which produce large masses of vegetative hyphae and asexual spores (conidia). Achieving a balance between vegetative growth and spore production is essential for successful and cost effective biocontrol. Low sporulation rates in the field can result in poor establishment and survival, whereas biocontrol activity occurs during the mycelial phase only. Commercial BCA products involve bulk preparations of conidia, however considerable variability in conidiation rates exists between BCAs, which can restrict the choice of strain for production. This study provides significant insight into the environmental factors critical for conidiation and secondary metabolite production and will lead to recommendations for local producers and users of *Trichoderma* BCAs.

5.1 Low pH and Nitrogen Source

The local pH and buffering capacity, in combination with the source and amount of nitrogen in the environment, are major influences in determining the fate of a hyphal cell. While it has been reported that in *Trichoderma*, photoconidiation occurs in a ring at the perimeter of the colony, in Chapter 3, both rings and disks of conidia were produced. Appearance of a disk was promoted by lowering the pH of the medium and by using the primary nitrogen sources glutamine and urea. Nitrogen and pH effects on conidiation were strongly influenced by the buffering state of the medium and in addition, conidial responses in the pH and nitrogen experiments were isolate/species specific.

Low pH and/or primary nitrogen sources promote photoconidiation and growth in species of *Trichoderma*, which has relevance for BCA production. Addition of primary nitrogen sources to the growth medium should increase photoconidiation in some isolates, however the additional cost of adding sufficient primary nitrogen may be prohibitive in some cases. Reducing the starting pH of the medium is comparably cheaper than adding extra nitrogen and, on the basis of these studies, generally more likely to promote conidiation. It is recommended that *Trichoderma* BCAs be screened to determine their optimum pH values for conidiation. The pH of the growth medium

should be acidified to the optimum pH of each isolate before inoculation. The medium should be unbuffered and constant light should also be employed throughout incubation to maximise conidiation.

Successful biocontrol in the soil is dependent upon the activities of the mycelial phase of the fungus, including antibiosis and mycoparasitism. In Chapters 2 and 3 a yellow pigment was reported in the medium of *T. harzianum* LU675 cultures. This yellow pigment has recently been identified as the anthraquinone pachybasin (Murray Munro, School of Biological Sciences, University of Canterbury), which is a secondary metabolite with antimicrobial activity (Donnelly & Sheridan, 1986, Reino *et al.*, 2007). Production of pachybasin was shown to be pH-dependent (Chapter 2, Sections C & D) and in *T. harzianum* CECT 2413, production of an α-pyrone with antimicrobial properties has been linked to Pac1 regulation (Moreno-Mateos *et al.*, 2007). pH regulation and control by PacC of antibiotic production and other secondary metabolites has been demonstrated in multiple fungal species, suggesting a common link between secondary metabolism and PacC regulation (Yu & Keller, 2005) Expression of the mycoparasitism-related gene encoding the chitinase Chit42 was also pH-dependent in *T. harzianum* CECT 2413 and *pac1* mutational studies suggested positive regulation by Pac1. Conserved PacC binding motifs have been identified in the upstream regions of *chit42* from both *T. hamatum* and *T. atroviride* (Steyaert *et al.*, 2004, 2003). Clearly a relationship exists between pH regulation and biocontrol mechanisms in *Trichoderma* spp..

The soil is a highly buffered environment, and acidic soils are often not conducive to horticultural cropping. Buffering of the medium in Chapter 2, negatively affected growth, conidiation and pachybasin production. It is possible that buffering may also affect expression of mycoparasitism genes. Soil pH and buffering capacity likely play a crucial role in determining successful biocontrol. *Trichoderma* strains which control disease well *in vitro* may not perform when applied in soils of a different pH and/or buffering capacity to the *in vitro* study. Either, existing *Trichoderma* BCAs should be screened and targeted to soils of the appropriate pH conditions, or, new isolates should be selected on the basis of capacity to antagonise pathogens under a given range of conditions. It may also be possible to create designer BCAs, which incorporate desired traits through protoplast fusion or genetic modification.

5.2 Circadian Rhythms

In this study, rhythmic conidiation and a rhythm in *gpd* expression were observed in isolates of *Trichoderma*, which strongly suggests the presence of a circadian rhythm (Chapters 2 & 3). While the exact nature of this rhythm is still to be resolved, these studies have implications for morphogenic and molecular studies in *Trichoderma* spp.. In addition to rhythmic conidiation, other processes and pathways within the cell are also likely to be influenced by the circadian rhythm. In some experiments, the secondary metabolite pachybasin appeared in bands on the filter paper (Section D, Figure 2.26), which suggests that pachybasin production may be rhythmic under some circumstances. As discussed above, the production of secondary metabolites, such as antibiotics, is important for biocontrol activity. It is possible that circadian rhythms may be associated with aspects of biocontrol activity.

Endogenous rhythms associated with morphogenesis, metabolism and perhaps antagonism, may affect the results from *in vitro* experiments which run for 2 d or longer. If sampling is done on a daily basis and cultures are grown in constant conditions, then it may be necessary to adjust sampling times to the endogenous clock. Consideration of the clock is likely also important for field experiments, where the soil abiotic factors, in combination with natural light, will determine rhythmic influences; particularly when sampling for gene expression studies. It may be necessary to incorporate controls within experiments which will distinguish between trends in response to the experimental system and circadian rhythms.

5.3 Experimental Variability

In Chapter 2 (Section C), phenotypic differences associated with the use of either conidial suspensions or mycelial plugs as inoculum were observed in both *T. atroviride* and *T. harzianum*. It was also observed in preliminary studies that *T. hamatum* master cultures older than 3 d resulted in reduced photoconidiation on broth-soaked filter (data not shown). For this reason, 2 d old cultures only were used to inoculate experiments. Whether this variability is due to the age of the inoculum or the effect of endogenous rhythms is not known. Variability in conidiation was also observed in preliminary experiments when culture plates were sealed with plastic wrap or media was sterilised

in the autoclave. On the basis of the observations in this study, the following recommendations are made for *in vitro* assays involving *Trichoderma* spp.:

1. Use conidial inoculum, or mycelial inoculum from 2 day old dark-grown cultures
2. Leave plates unsealed or use paper tape to allow air diffusion.
3. Prepare media in a stove-top pressure cooker
4. Where possible, filter sterilise nitrogen and carbon sources and add prior to pouring plates
5. For maximum photoinduction of conidiation, place plates under constant light immediately following inoculation
6. For maximum injury-induction of conidiation, cultures should be allowed to reach the edge of the plate before injuring.

5.4 *Trichoderma* as a Molecular Model for Conidiation

Multiple orthologues of conidiation genes from the filamentous fungal models, *N. crassa* and *A. nidulans*, were investigated in this study and shown to have high sequence similarity, such as *RcoT*, and, in the case of *blr2*, functional similarity. These studies have highlighted the commonalities between the filamentous fungi and further validate the use of *Trichoderma* as a molecular model for conidiation studies.

5.5 Future Directions

These studies have provided insight into two key abiotic factors regulating conidiation: the nitrogen source and ambient pH. Future research on these key factors is required to understand the mechanisms behind their regulation of conidiation. The results in Chapter 2 Sections B and C, strongly suggest cross-regulation between primary nitrogen and low pH promotion of conidiation in *T. atroviride*. It was proposed that low pH promotes conidiation, in part, by increasing the availability of primary nitrogen sources (Chapter 2, Section C). This could be tested by investigating photoconidiation at a range of pH values and nitrogen concentrations. If the absence of conidiation in *T. atroviride* LU298 at higher pH values on buffered media is associated with a decrease in N availability, then addition of excess nitrogen may promote conidiation at higher pH values.

In each of the nitrogen and pH experiments, cultures responded to light or injury stimuli in an isolate-specific fashion. Each isolate in this study represented a different species, which suggests that some of the observations made in this study may be species-specific. Evaluating multiple isolates of a single species in conidiation assays on variable nitrogen and pH should identify whether species-specific parameters exist

The *T. harzianum* isolate in this study excreted large amounts of the anthraquinone pachybasin. Pachybasin has been reported to have antimicrobial activity, however only a few studies have been undertaken. In a recent screen of *T. harzianum* isolates from the Lincoln University culture collection, this isolate was identified as being a high producer of pachybasin. High production of an antimicrobial secondary metabolite may enhance biocontrol activity. The antibiosis activity of this isolate could be tested to determine whether pachybasin producing strains have relevance for biocontrol.

It is evident from this study that conidiation can be rhythmic in species of *Trichoderma*, however the exact nature of the rhythm is still to be defined. The *T. harzianum* isolate used in this study has great potential as a model for circadian responses in *Trichoderma*. Using specially designed race-tubes, the period of the rhythm could be defined which would create a system in which to test predictions on genes involved in rhythmic behaviour, such as the *blr* genes and *frq*.

Genomic analysis identified a conserved *erg3* gene downstream from *rcoT* and orthologues in the ascomycetous filamentous fungi, but not in the ascomycetous yeasts or the basidiomycetes. Dispruption of *rcoT* orthologues may have had a cis-effect on *erg3* expression, which contributed to the gross deficiencies seen in the resulting phenotypes. This could be tested by examining *erg3* expression in the *rco-1*, *rcoA* and *tupA* loss of function mutants.

Analysis of the upstream regions of *rcoT* and orthologues revealed multiple Cre1 and C_4T binding motifs suggesting regulation by carbon catabolite repression and stress. In addition, PacC motifs were present in all sequences examined, except for *N. crassa*. No major expressional analysis of the *rco-1*, *rcoA* and *tupA* genes has been undertaken. Expressional analysis of *rcoT* in the presence of different carbon sources, in response to stress and at variable pH levels is required to determine if *rcoT* undergoes transcriptional regulation induced by these factors.

The mutational study described in Chapter four was unsuccessful. To determine whether RcoT production was reduced in the putative antisense transformants, protein analysis will need to be undertaken. An alternative approach to assigning a role for this gene would be to create partial loss of function mutants, in which specific domains are removed, such as the sequential loss of the WD-repeats. Comparison of gene expression profiles between the wild-type and mutants would identify genes regulated by RcoT.

Despite the wealth of research on the topic, much is still to be understood about the regulatory pathways determining conidiogenesis of a hyphal cell. These studies have provided further insight into morphogenesis and the molecular mechanisms underpinning conidiation, while highlighting additional complexities. The work presented in this thesis will significantly contribute to the body of literature on fungal conidiation.

Chapter Six
References

Adams, T.H., Wieser, J.K. & Yu, J. (1998). Asexual sporulation in *Aspergillus nidulans*. *Microbiology and Molecular Biology Reviews* **62:** (1) pp. 35-54.

Agrios, G.N. (2005). *Plant Pathology, 5th edition*. Academic Press Inc., U.S.A..

Alcazar-Fuoli, L., Mellado, E., Garcia-Effron, G., Buitrago, M.J., Lopez, J.F., Grimalt, J.O., Cuenca-Estrella, J.M. & Rodriguez-Tudela, J.L. (2006). *Aspergillus fumigatus* C-5 sterol desaturases Erg3A and Erg3B: Role in sterol biosynthesis and antifungal drug susceptibility. *Antimicrobial Agents and Chemotherapy* **50:** (2) pp. 453-460.

Arpaia, G., Cerri, F., Baima, S. & Macino, G. (1999). Involvement of protein kinase C in the response of *Neurospora crassa* to blue light. *Molecular and General Genetics* **262:** pp. 314-322.

Aube, C. & Gagnon, C. (1969). Effect of carbon and nitrogen nutrition on growth and sporulation of *Trichoderma viride* Pers. ex Fries. *Canadian Journal of Microbiology* **15:** pp. 703-706.

Awuah, R.T. & Lorbeer, J.W. (1989). Role of light, temperature, and method of propagation in cultural variability of *Fusarium oxysporum* f.sp. *apii* race 2. *Mycologia* **81:** (2) pp. 278-283.

Ballario, P., Vittorioso, P., Magrelli, A., Talora, C., Cabibbo, A. & Macino, G. (1996). White-collar-1, a central regulator of blue light responses in *Neurospora*, is a zinc finger protein. *The EMBO Journal* **15:** (7) pp. 1650-1657.

Bastos, C.N. (2001). Effect of temperature, pH and nutrition on growth and sporulation of *Trichoderma stromaticum* sp. nov., an antagonist of cocoa witches' broom pathogen. *Summa Phytopathologica* **27:** (1) pp. 73-76.

Baum, D. & Horwitz, B.A. (1991). Changes in synthesis and abundance of specific polypeptides at early and late stages of blue-light-induced sporulation of *Trichoderma*. *Journal of Photochemistry and Photobiology* **11:** pp. 117-127.

Berlin, V., & Yanofsky, C. (1985). Isolation and characterisation of genes differentially expressed during conidiation of *Neurospora crassa*. *Molecular and Cellualr Biology* **5:** pp. 849-855.

Berrocal-Tito, G., Sametz-Baron, L., Eichenburg, K., Horwitz, B.A. & Herrera-Estrella, A. (1999). Rapid blue light regulation of a *Trichoderma harzianum* photolyase gene. *The Journal of Biological Chemistry* **247:** (20) pp. 14288-14294.

Berrocal-Tito, G., Rosales-Saavedra. T., Herrera-Estrella, A. & Horwitz, B.A. (2000). Characterisation of blue-light and developmental regulation of the photolyase ghene *phr1* in *Trichoderma harzianum. Photochemistry and Photobiology* **71:** (5) pp. 662-668.

Betina, V. (1995). Photoinduced conidiation in *Trichoderma viride*. *Folia Microbiologica* **40:** pp. 219-224.

Betina, V. & Farkaš V. (1998). Sporulation and light-induced development in *Trichoderma*. pp. 75-94 in *Trichoderma and Gliocladium Volume 1*, editors G.E. Harman and C.P. Kubicek. Taylor and Francis Ltd., London.

Betina, V. & Zajacová, J. (1978a). Regulation of periodicity and intensity of photo-induced condidiation of *Trichoderma viride*. *Folia Microbiologica* **23:** pp. 453-459.

Betina, V. & Zajacová, J. (1978b). Inhibition of photo-induced *Trichoderma viride* conidiation by RNA synthesis inhibitors. *Folia Microbiologica* **23:** pp. 460-464.

Bissett, J. (1991). A revision of the genus *Trichoderma*. III. Section *Pachybasium*. *Canadian Journal of Botany* **69:** pp. 2372-2417.

Bok, J.W. & Keller, N.P. (2004). LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryotic Cell* **3:** (2) pp. 527-535.

Braun, B. R. & Johnson, A.D. (1997). Control of filament formation in *Candida albicans* by the transcriptional repressor Tup1. *Science* **277:** pp. 105-109.

Brian, P.W. & Hemming, H.G. (1950). Some nutritional conditions affecting spore production by *Trichoderm viride* pers. ex Fries. *Transactions of the British Mycological society* **33:** pp. 132-141.

Casas-Flores, S., Rios-Momberg, M., Bibbens, M., Ponce-Noyola, P. & Herrera-Estrella, A. (2004). BLR-1 and BLR-2, key regulatory elements of photoconidiation and mycelial growth in *Trichoderma atroviride*. *Microbiology* **150:** pp. 3561-3569.

Casas-Flores, S., Rios-Momberg, M, Rosales-Saavedra, T., Martinez-Hernandez, P. Olmedo-Monfil, V. & Herrera-Estrella, A. (2006). Cross talk between a fungal blue-light perception system and the cyclic AMP signalling pathway. *Eukaryotic Cell* **5:** (3) pp. 499-506.

Caspani, G., Tortora, P., Hanozet, G.M. & Guerritore, A. (1985). Glucose-stimulated cAMP increase may be mediated by intracellular acidification in *Saccharomyces cerevisiae*

Chalot, M. & Brun, A. (1998). Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas. *FEMS Microbiology Reviews* **22:** pp. 21-44.

Chernin, L. & Chet, I. (2002). Microbial enzymes in the biocontrol of plant pathogens and pests. pp. 171-226 in *Enymes in the Environment: Activity, Ecology and Applications*, editors R.G. Burns and R.P. Dick. Marecl Dekker, Inc., New York.

Christensen, M.K., Falkeid, G., Loros, J.J., Dunlap, J.C., Lillo, C. & Ruoff, P. (2004). A nitrate-induced *frq*-less oscillator in *Neurospora crassa*. *Journal of Biological Rhythms* **19:** (4) pp. 280-286.

Collett, M.A., Garceau, N., Dunlap, J.C. & Loros, J.J. (2002). Light and clock expression of the Neurospora clock gene *frequency* is differentially driven by but dependent on WHITE COLLAR-2. *Genetics* **160:** pp. 149-158.

Conlan, R.S., Gounalaki, N., Hatzis, P. & Tzamarias, D. (1999). The Tup1-Cyc8 protein complex can shift from a transcriptional co-repressor to a transcriptional co-activator. *Journal of Biological Chemistry* **274:** (1) pp. 205-210.

Cziferszky, A., Mach, R.L. & Kubicek, C.P. (2002). Phosphorylation positively regulates DNA binding of the carbon catabolite repressor Cre1 of *Hypocrea jecorina. Journal of Biological Chemistry* **277:** (17) pp. 14688-14694.

D'Andrea, L.D. & Regan, L. (2003). TPR proteins: The versatile helix. *Trends in Biochemical Sciences* **28:** (12) pp. 655-662.

Deitzer, G.F., Horwitz, B.A. & Gressel, J. (1988). Rhythms in blue-light-induced conidiation of a wild type and a mutant strain of *Trichoderma harzianum*. *Photochemistry and Photobiology* **47:** (3) pp. 425-431.

Donaton, M.C.V., Holsbeeks, I., Lagatio, O., Van Zeebroeck, G., Crauwels, M., Winderickx, J. & Thevelain, J.M. (2003). The Gap1 general amino acid permease acts as an amino acid sensor for activation of protein kinase A targets in the yeast *Saccharomyces cerevisiae*. *Molecular Microbiology* **50:** (3) pp. 911-929.

Drori, N., Kramer-Haimovich, H., Rollins, J., Dinoor, A., Okon, Y., Pines, O. & Prusky, D. (2003). External pH and nitrogen source affect secretion of pectate lyase by *Colletotrichum gloeosporioides*. *Applied and Environmental Microbiology* **69:** (6) pp. 3258-3262.

El-Hasan, A., Walker. F., Schone, J. & Buchenauer, H. (2007). Antagonistic effect of 6-pentyl-alpha-pyrone produced by *Trichoderma harzianum* toward *Fusarium moniliforme*. *Journal of Plant Diseases and Protection* **114:** (2) pp. 62-68.

Ellison, P.J., Harrower, K.M., Chilvers, G.A. & Owens, J.D. (1981). Patterns of sporulation in *Trichoderma viride*. *Transactions of the British Mycological Society* **76:** (3) pp 441-445.

Eshel, d., Miyara, I., Ailing, T., Dinoor, A. & Prusky, D. (2002). pH regulates endoglucanase expresseion and virulence of *Alternaria alternate* in persimmon fruit. *Molecular Plant-Microbe Interactions* **15:** (8) pp. 774-779.

Froehlich, A.C., Liu, Y., Loros, J.J. & Dunlap, J.C. (2002). White Collar-1, a circadian blue light receptor, binding to the *frequency* promoter. *Science* **297:** pp. 815-819.

Franchi, L., Fulci, V. & Macino, G. (2005). Protein kinase C modulates light responses in *Neurospora* by regulating the blue light photoreceptor WC-1. *Molecular Microbiology* **56:** (2) pp. 334-345.

Galun, E. (1971). Morphogenesis in *Trichoderma*: Induction of conidiation by narrow-beam illumination of restricted areas of the fungal colony. *Plant and Cell Physiology* **12:** pp. 779-783.

Gams, W. & Bissett, J. (1998). Morphology and identification of *Trichoderma*. pp. 3-34 in *Trichoderma and Gliocladium Volume 1*, editors G.E. Harman and C.P. Kubicek. Taylor and Francis Ltd., London.

Garrison, R.G. & Boyd, K.S. (1973). Dimorphism of *Penicillium marneffei* as observed by electron microscopy. *Canadian Journal of Microbiology* **19:** pp. 1305-1309.

Gao, L., Sun, M.H., Liu, X.Z. & Che, Y.S. (2007). Effects of carbon concentration and carbon to nitrogen ratio on the growth and sporulation of several biocontrol fungi. *Mycological Research* **111:** pp. 87-92.

Ghisalberti, E.L., Narbey, M.J., Dewan, M.M. & Sivasithamparam, K. (1990). Variability among strains of *Trichoderma harzianum* in their ability to reduce take-all and to produce pyrones. *Plant and Soil* **121:** pp. 287-291.

Ghisalberti, E.L. & Sivasithamparam, K. (1991). Antifungal antibiotics produced by *Trichoderma* spp.. *Soil Biology and Biochemistry* **23:** (11) pp. 1011-1020.

Gradišnik-Grapulin, M. & Legiša, M. (1997). A spontaneous change in the intracellular cyclic AMP level in *Aspergillus niger* is influenced by the sucrose concentration in the medium and by light. *Applied and Environmental Microbiology* **63:** (7) pp. 2844-2849.

Greene, A.V., Keller, N., Haas, H. & Bell-Pederson, D. (2003). A circadian oscillator in *Aspergillus* spp. regulates daily development and gene expression. *Eukaryotic Cell* **2:** pp. 231-237.

Grešík, M., Kolarova, N. & Farkaš, V. (1988). Membrane potential, ATP and cyclic AMP changes induced by light in *Trichoderma viride*. *Experimental Mycology* **12:** pp. 295-301.

Grešík, M., Kolarova, N. & Farkaš, V. (1991). Hyperpolarization and intracellular acidification in *Trichoderma viride* as a response to illumination. *Journal of General Microbiology* **137:** pp. 2605-2609.

Gressel, J., Bar-Lev, S. & Galun, E. (1975). Blue light induced response in the absence of free oxygen. *Plant and Cell Physiology* **16:** pp. 367-370.

Gressel, J. & Galun, E. (1967). Morphogenesis in *Trichoderma*: Photoinduction and RNA. *Developmental Biology* **15:** pp 575-598.

Gressel, J.B. & Hartmann, K.M. (1968). Morphogenesis in *Trichoderma*: Action spectrum of photoinduced sporulation. *Planta* **79:** pp. 271-274.

Gutter, Y. (1957). Effect of light on sporulation of *Trichoderma viride* pers. ex Fries. *Bulletin of the Research Council of Israel* **5D:** pp. 273-286.

He, Q., Shu, H., Cheng, P., Chen, S., Wang, L. & Lui, Y. (2005). Light-independent phosphorylation of WHITE COLLAR-1 regulates its function in the *Neurospora* circadian negative feedback loop. *The Journal of Biological Chemistry* **280:** (17) pp. 17526-17532.

He, Q. & Lui, Y. (2005). Molecular mechanism of light responses in *Neurospora*: from light-induced transcription to photoadaptation. *Genes and Development* **19:** pp. 2888-2899.

Herrera-Estrella, A. & Horwitz, B.A. (2007). Looking through the eyes of fungi: Molecular genetics of photoreception. *Molecular Microbiology* **64:** (1) pp. 5-15.

Hickman, M.J. & Winston, F. (2007). Heme levels switch the function of Hap1 of *Saccharomyces cerevisiae* between transcriptional activator and transcriptional repressor. *Molecular and Cellular Biology* **27:** (21) pp. 7414-7424.

Hicks, J., Lockington, R.A., Strauss, J., Dieringer, D., Kubicek, C.P., Kelly, J. & Keller, N. (2001). RcoA has pleiotropic effects on *Aspergillus nidulans* cellular development. *Molecular Microbiology* **39:** (6) pp. 1482-1493.

Horák, J. (1997). Yeast nutrient transporters. *Biochimica et Biophysica Acta* **1331:** pp. 41-79.

Horwitz, B.A., Gressel, J. & Malkin, S. (1985a). Photoperception mutants in *Trichoderma* : Mutants that sporulate in response to stress but not light. *Current Genetics* **9:** pp. 605-613.

Horwitz, B.A., Gressel, J., Malkin, S. & Epel, B.L. (1985b). Modified cryptochrome *in vivo* absorption in *dim* photosporulation mutants of *Trichoderma*. *Proceedings of the National Academy of Sciences U.S.A.* **82:** pp. 2736-2740.

Horwitz, B.A., Perlman, A. & Gressel, J. (1990). Induction of *Trichoderma* sporulation by nanosecond laser pulses: Evidence against cryptochrome cycling. *Photochemistry and Photobiology* **51:** (1) pp. 99-104.

Jabet, C., Sprague, E.R., VanDemark, A.P. & Wolberger, C. (2000). Characterization of the N-terminal domain of the yeast transcriptional repressor Tup1. Proposal for an association model of the repressor complex Tup1-Ssn6. *Journal of Biological Chemistry* **275:** pp. 9011-9018.

Jackson, A.M., Whipps, J.M. & Lynch, J.M. (1991). Nutritional studies of four fungi with disease biocontrol potential. *Enzyme and Microbial Technology* **13:** pp. 456-461.

Kindermann, J., El-Ayouti, Y., Samuels, G.J. & Kubicek, C.P. (1998). Phylogeny of the genus *Trichoderma* based on sequence analysis of the internal transcribed spacer region 1 of the rDNA cluster. *Fungal Genetics and Biology* **24:** pp. 298-309.

Klein, D. & Eveleigh, D.E. (1998). Ecology of Trichoderma. pp. 57-74 in *Trichoderma and Gliocladium Volume 1*, editors G.E. Harman and C.P. Kubicek. Taylor and Francis Ltd., London.

Knudsen, G.R., Eschen, D.J., Dandurand, L.M. & Wang, Z.M. (1991). Method to enhance growth and sporulation of pelletized biocontrol fungi. *Applied and Environmental Microbiology* **57:** (10) pp. 2864-2867.

Kredics, L., Manczinger, L., Antal, Z., Pénzea, Z., Szekeres, A., Kevei, F. & Nagy, E. (2004). *In vitro* water activity and pH dependence of mycelial growth and extracellular enzyme activities of *Trichoderma* strains with biocontrol potential. *Journal of Applied Microbiology* **96:** pp. 491-498.

Kullnig, C.M., Krupica, T., Woo, S.L., Mach, R.L., Rey, M., Benítez, T., Lorito, M. & Kubicek, C.P. (2001). Confusion abounds over identities of *Trichoderma* biocontrol isolates. *Mycological Research* **105:** (7) pp. 770-772.

Kumagai, T. & Oda, Y. (1969). An action spectrum for photoinduced sporulation in the fungus *Trichoderma viride*. *Plant and Cell Physiology* **10:** pp. 387-392.

Lakin-Thomas, P.L. & Brody, S. (2004). Circadian rhythms in microorganisms: New complexities. *Annual Review of Microbiology* **58:** pp. 489-519.

Lee, N., D'Souza, C.A. & Kronstad, J.W. (2003). Of smuts, blasts, mildews and blights: cAMP signalling in phytopathogenic fungi. *Annual Review of Phytopathology* **41:** pp. 399-427.

Lee, K. & Ebbole, D.J. (1998). Tissue-specific repression of starvation and stress responses of the *Neurospora crassa con-10* gene is mediated by Rco1. *Fungal Genetics and Biology* **23:** pp. 269-278.

Lendenfield, T. Kubicek, C.P. (1998). Characterization and properties of protein kinase C from the filamentous fungus *Trichoderma reesei*. *Biochemical Journal* **330:** pp. 689-694.

Lewis, J.A. & Papavizas, G.C. (1983). Production of chlamydospores and conidia by *Trichoderma* spp. in liquid and solid growth media. *Soil Biology and Biochemistry* **15:** (3) pp. 351-357.

Lieckfeldt, E., Samuals, G.J., Nirenberg, H.I. & Petrini, O. (1999). A morphological and molecular perspective of *Trichoderma viride*: Is it one or two species?. *Applied and Environmental Microbiology* **65:** (6) 2416-2428.

Linden, H., Ballerio, P. & Macino, G. (1997). Blue light regulation in *Neurospora crassa*. *Fungal Genetics and Biology* **22:** pp. 141-150.

Linden, H. & Macino, G. (1997). White collar 2, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*. *The EMBO Journal* **16:** (1) pp. 98-109.

Lombardi, L.M. & Brody, S. (2005). Circadian rhythms in *Neurospora crassa*: Clock gene homologues in fungi. *Fungal Genetics and Biology* **42:** pp. 887-892.

Loros, J.J. & Dunlap, J.C. (1991). *Neurospora crassa* clock-controlled genes are regulated at the level of transcription. *Molecular and Cellular Biology* **11:** (1) pp. 558-563.

Loros, J.J. & Dunlap, J.C. (2001). Genetic and molecular analysis of circadian rhythms in *Neurospora*. *Annual Review of Physiology* **63:** 757-794.

Loubradou, G., Brachmann, A., Feldbrügge, M. & Hahmann, R. (2001). A homologue of the transcriptional repressor Ssn6p antagonises cAMP signalling in *Ustilago maydis*. *Molecular Microbiology* **40:** (3) pp. 719-730.

McIlvaine, T.C. (1921). A buffer solution for colorimetric comparison. *Journal of Biological Chemistry* **49:** pp. 183-186.

Madi, L., Ebbole, D.J., White, B.T. & Yanofsky, C. (1994). Mutants of *Neurospora crassa* that alter gene expression and conidia development. *Proceedings of the National Academy of Sciences U.S.A.* **91:** pp. 6226-6230.

Malavé, T.M. & Dent, S.Y.R. (2006). Transcriptional repression by Tup1-Ssn6. *Biochemical and Cellular Biology* **4:** pp. 43-443.

Marzluf, G.A. (1997). Genetic regulation of nitrogen metabolism in the fungi. *Microbiology and Molecular Biology Reviews* **61:** (1) pp. 17-32.

Miller, J.J. & Reid, J. (1961). Stimulation by light of sporulation in *Trichoderma lignorum* (Tode) Harz. *Canadian Journal of Botany* **39:** pp. 259-262.

Mitchell, P. (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* **191:** pp. 144-148.

Monga, D. (2001). Effect of carbon and nitrogen sources on spore germination, bio-mass production and antifungal metabolites by species of *Trichoderma* and *Gliocladium*. *Indian Phytopathology* **54:** (4) pp. 435-437.

Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press, Cambridge.

Moreno-Mateos, M.A., Delgado-Jarana, J., Codón, A.C. & Benítez, T. (2007). pH and Pac1 control development and antifungal activity in *Trichoderma harzianum*. *Fungal Genetics and Biology* **44:** (12) pp. 1355-1367.

Mukherjee, M., Mukherjee, P.K. & Kale, S.P. (2007). cAMP signalling is involved in growth, germination, mycoparasitism and secondary metabolism in *Trichoderma virens*. *Microbiology* **153:** (6) pp. 1734-1742.

Mukherjee, P.K., Latha, J., Hadar, R. & Horwitz, B.A. (2003). TmkA, a mitogen-activated protein kinase of *Trichoderma virens*, is involved in biocontrol properties and repression of conidiation in the dark. *Eukaryotic Cell* **2:** (3) pp. 446-455.

Mukherjee, P.K. Latha, J., Hadar, R. & Horwitz, B.A. (2004). Role of two G-protein alpha subunits, TgaA and TgaB, in the antagonism of plant pathogens by *Trichoderma virens*. *Applied and Environmental Microbiology* **70:** (1) pp. 542-549.

Neer, E.J., Schmidt, C.J., Nambudripad, R. & Smith, T.F. (1994). The ancient regulatory-protein family of WD-repeat proteins. *Nature* **31:** pp. 297-300.

Nemcovic, M. & Farkaš, V. (1998). Stimulation of conidiation by derivatives of cAMP in *Trichoderma viride*. *Folia Microbiologica* **43:** (4) pp. 399-402.

Nielson, K. & Heitman, J. (2007). Sex and Virulence of Human Pathogenic Fungi. *Advances in Genetics* **57:** pp. 143-173.

Ninnemann, H. (2001). Developments in blue light reception/signal transduction in fungal morphogenesis: *Neurospora*/*Phycomyces*, nitrate reductase and NO-synthase. *Recent Research Developments in Plant Biology* **1:** pp. 105-130.

Nussinov, R. (1990). Sequence signals in eukaryotic upstream regions. *Critical Reviews in Biochemistry and Molecular Biology* **25:** (3) pp. 185-24.

Olsson, S. (1999). Nutrient translocation and electrical signalling. pp. 25-48 in *The Fungal Colony*, editors N.A.R. Gow, G.D. Robson and G.M. Gadd. Cambridge University Press, Cambridge.

Pall, M.L. (1977). Cyclic AMP and the plasma membrane potential in *Neurospora crassa*. *Journal of Biological Chemistry* **252:** pp. 462-480.

Papavizas, G.C. (1985). *Trichoderma* and *Gliocladium*: Biology, ecology, and potential for biocontrol. *Annual Review of Phytopathology* **23:** pp. 23-54.

Parks, L.W. & Casey, W.M. (1995). Physiological implications of sterol biosynthesis in yeast. *Annual Review of Microbiology* **49:** pp. 95-116.

Peñalva, M.A. & Arst, Jr., H.N. (2002). Regulation of gene expression by ambient pH in filamentous fungi and yeasts. *Microbiology and Molecular Biology Reviews* **66:** (3) pp. 426-446.

Peñalva, M.A. & Arst, Jr., H.N. (2004). Recent advances in the characterization of ambient pH regulation of gene expression in filamentous fungi and yeasts. *Annual Review of Microbiology* **58:** pp. 425-451.

Peterbauer, C.K., Litscher, D. & Kubicek, C.P. (2002). The *Trichoderma atroviride seb1* (stress response element binding) gene encodes an AGGGG-binding protein which is involved in the response to high osmolarity stress. *Molecular Genetics and Genomics* **268:** pp. 223-231.

Prusky, D. 7 Yakoby, N. (2003). Pathogenic fungi: Leading or led by ambient pH. *Molecular Plant Pathology* **4:** (6) pp. 509-516.

Puyesky, M., Ponce-Noyola, P., Horwitz, B.A. & Herrera-Estrella, A. (1997). Glyceraldehyde-3-phosphate dehydrogenase expression in *Trichoderma harzianum* is repressed during conidiation and mycoparasitism. *Microbiology* **143:** pp. 3157-3164.

Ramsdale, M. (1999). Circadian rhythms in filamentous fungi. pp. 75-107 in *The Fungal Colony*, editors N.A.R. Gow, G.D. Robson and G.M. Gadd. Cambridge University Press, Cambridge.

Redd, M.J., Arnaud, M.B. & Johnson, A.D. (1997). A complex composed of tup1 and ssn6 represses transcription in vitro. *Journal of Biological Chemistry* **272:** 11193-11197.

Reino, J.L., Guerrero, R.F., Hernández-Galán, R. & Collado, I.G. (2007). Secondary metabolites from species of the biocontrol agent *Trichoderma*. *Phytochemistry Reviews*, DOI 10.1007/s11101-006-9032-2.

Rifai, M.A. (1969). A revision of the genus *Trichoderma*. *Mycological Papers* **116:** pp. 1-56.

Rocha-Ramírez, V., Omero, C., Chet, I., Horwitz, B.A. & Herrera-Estrella, A. (2002). *Trichoderma atroviride* G-protein α-subunit gene *tga1* is involved in mycoparasitic coiling and conidiation. *Eukaryotic Cell* **1:** (4) pp. 594-605.

Rosales-Saavedra, T., Esquivel-Naranjo, E.U., Casas-Flores, S., Martínez-Hernández, P., Ibarra-Laclette, E., Cortes-Penagos, C. & Herrera-Estrella, A. (2006). Novel light-regulated genes in *Trichoderma atroviride*: a dissection by cDNA microarrays. *Microbiology* **152:** pp. 3305-3317.

Rosenberg, G.B. & Pall, M.L. (1983). Characterization of an ATP-Mg2+-dependent guanine nucleotide-stimualted adenylate cyclase from *Neurospora crassa. Archives of Biochemistry and Biophysics* **221:** pp. 243-253.

St Leger, R.J., Nelson, J.O. & Screen, S.E. (1999). The entomopathogenic fungus *Metarhizium anisopliae* alters ambient pH, allowing extracellular protease production and activity. *Microbiology* **145:** pp. 2691-2699.

Sargent, M.L., Briggs, W.R. & Woodward, D.O. (1966). The circadian nature of a rhythm expressed by an invertaseless strain of *Neurospora crassa*. *Plant Physiology* **41:** pp. 1343-1349.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edition. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press.

Sametz-Baron, L., Berrocal-Tito, G.M., Amit, R., Herrera-Estrella, A. & Horwitz, B.A. (1997). Photreactivation of UV-inactivated spores of *Trichoderma harzianum*. *Photochemistry and Photobiology* **65:** (5) pp. 849-854.

Samuels, G.J. (1996). *Trichoderma*: A review of biology and systematics of the genus. *Mycological Research* **100:** pp. 923-935.

Schaeffer, H.J. & Weber, M.J. (1999). Mitogen-activated protein kinases: Specific messages from ubiquitous messengers. *Molecular and Cellular Biology* **19:** (4) pp. 2435-2444.

Schultz, J., Marshall-Carlson, L. & Carlson, M. (1990). The N-terminal TPR region is the functional domain of SSN6, a nuclear phosphoprotein of *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **10:** (9) pp. 4744-4756.

Schmoll, M., Franchi, L. & Kubicek, C.P. (2005). Envoy, a PAS/LOV domain protein of *Hypocrea jecorina* (anamorph *Trichoderma reesei*), modulates cellulase gene transcription in response to light. *Eukaryotic Cell* **4:** (12) pp. 1998-2007.

Schrüfer, K. & Lysek, G. (1990). Rhythmic growth and sporulation in *Trichoderma* species: Differences within a population of isolates. *Mycological Research* **94:** (1) pp. 124-127.

Schwerdtfeger, C. & Linden, H. (2001). Blue light adaptation and densensitization of light signal transduction in *Neurospora crassa* **39:** (4) pp. 1080-1087.

Schwerdtfeger, C. & Linden, H. (2003). VIVID is a flavoprotein and serves as a fungal blue light photoreceptor for photoadaptation. *The EMBO Journal* **22:** (18) pp. 4846-4855.

Shinohara, M.L., Loros, J.J. & Dunlap, J.C. (1998). Glyceraldehyde-3-phosphate dehydrogenase is regulated on a daily basis by the circadian clock. *The Journal of Biological Chemistry* **272:** (1) pp. 446-452.

Sivasithamparam, K. & Ghisalberti, E.L. (1998). Secondary Metabolism in *Trichoderma* and *Gliocladium* pp. 139-191 in *Trichoderma and Gliocladium Volume 1*, editors G.E. Harman and C.P. Kubicek. Taylor and Francis Ltd., London.

Smith, R.L. & Johnson, A.D. (2000). Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends in Biochemical Sciences*

Sokolovsky, V.Y., Lauter, F., Müller-Röber, B., Ricci, M., Schmidhauser, T.J. & Russo, E.A. (1992). Nitrogen regulation of blue light-inducible genes in *Neurospora crassa*. *Journal of General Microbiology* **138:** pp. 2045-2049.

Springer, M.L. & Yanofsky, C. (1992). Expression of *con* genes along the three sporulation pathways of *Neurospora crassa*. *Genes and Development* **6:** pp. 1052-1057.

Steyaert, J.M., Ridgway, H.J., Elad, Y. & Stewart, A. (2003). Genetic basis of mycoparasitism: A mechanism of biological control by species of *Trichoderma*. *New Zealand Journal of Crop and Horticultural Science* **31:** pp. 281-291.

Steyaert, J.M., Stewart, A., Jaspers, M.V., Carpenter, M., Ridgway, H.J. (2004). Co-expression of two genes, a chitinase (*chit42*) and proteinase (*prb1*), implicated in mycoparasitism by *Trichoderma hamatum. Mycologia* **96:** (6) pp. 1245-1252.

Sulová, Z. & Farkaš, V. (1991). Photoinduced conidiation in *Trichoderma viride*: A study with photoinhibitors. *Folia Microbiologica* **36:** (3) pp. 267-270.

Sulová, Z., Hrmová, M. & Farkaš, V. (1990). Photostimualted oxygen uptake in *Trichoderma viride*. *Journal or General Microbiology* **136:** pp. 2287-2290.

Szabo, R. & Stofaníková, V. (2002). Presence of organic sources of nitrogen is critical for filament formation and pH-dependent morphogenesis in *Yarrowia lipolytica*. *FEMS Microbiology Letters* **206:** (1) pp. 45-50.

Támová, G., Farkaš, V. & Betina, V. (1995). Relationship between light intensity and the changes in the intracellular level of ATP during photoinduction in *Trichoderma viride. Folia Microbiologica* **40:** (5) pp. 516-518.

Taylor, B.L. & Zhulin, I.B. (1999). PAS domains: Internal sensors of oxygen, redox potential and light. *Microbiology and Molecular Biology Reviews* **63:** pp. 479-506.

ter Schure, E.G., van Riel, N.A.W. & Verrips, C.T. (2000). The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Mircobiology Reviews* **24:** pp. 67-83.

Thevelein, J.M., Beullens, M., Honshoven, F., Hoebeeck, G., Detremerie, K., den Hollander, J.A. & Jans, A.W. (1987). Regulation of the cAMP level in the yeast *Saccharomyces cerevisiae*: Intracellular pH and the effect of membrane depolarizing compounds. *Journal of General Microbiology* **133:** (8) pp. 2191-2196

Thevelein, J.M., Geladé, R., Holsbeek, I., Lagatie, O., Popova, Y., Rolland, F., Stolz, F., Van de Velde, S., Van Dijck, P., Vandormael, P., Van Nuland, A., Van Roey, K., Van Zeebroeck, G. & Yan, B. (2005). Nutrient sensing systems for rapid activation of the protein kinase A pathway in yeast. *Biochemical Society Transactions* **33:** (1) pp. 253-256.

Todd, R.B., Greenhalgh, J.R., Hynes, M.J. & Andrianopoulos, A. (2003). TupA, the *Penicillium marneffei* Tup1p homologue, represses both yeast and spore development. *Molecular Microbiology* **48:** (1) pp. 85-94.

Tomeo, M.E., Fenner, G., Tove, S.R. & Parks, L.W. (1992). Effect of sterol alterations on conjugation in *Saccharomyces cerevisiae*. *Yeast* **8:** pp. 1015-1024.

Toyota, K., Onai, K. & Nakashima, H. (2002). A new *wc-1* mutant of *Neurospora crassa* shows unique light sensitivity in the circadian conidiation rhythm. *Molceular Genetics and Genomics* **268:** (1) pp. 56-61.

Treger, J.M., Magee, T.R. & McEntee, K. (1998). Functional analysis of the stress response element and its role in the multistress response of *Saccharomyces cerevisiae. Biochemical and Biophysical Research Communications* **243:** pp. 13-19.

Treitel, M.A. & Carlson, M. (1995). Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proceedings of the National Academy of Sciences U.S.A.* **92:** pp. 3132-3236.

Tzamarias, D. & Struhl, K. (1995). Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8-Tup1 corepressor complex to differentially regulated promoters. *Genes and Development* **9:** pp. 821-831.

Van Nuland, A., Vandormael, P., Donaton, M., Alenquer, M., Lourenço, A., Quintino, E., Versele, M. & Thevelein, J.M. (2006). Ammonium permease-based sensing mechanism for rapid ammonium activation of the protein kinase A pathway in yeast. *Molecular Microbiology* **59:** (5) pp. 1485-1505.

Vinale, F., Marra, R., Scala, F., Ghisalberti, E.L., Lorito, M. & Sivasithamparm, K. (2006). Major secondary metabolites produced by two different *Trichoderma* strains active against different phytopathogens. *Letters in Applied Microbology* **43:** (2) pp. 143-148.

Watkinson, S. (1999). Metabolism and hyphal differentiation in large basidiomycete colonies. pp. 126-156 in *The Fungal Colony*, editors N.A.R. Gow, G.D. Robson and G.M. Gadd. Cambridge University Press, Cambridge.

Wiame, J.M., Grenson, M. & Arst Jr., H.N. (1985). Nitrogen catabolite repression in yeast and filamentous fungi. *Advances in Microbiology and Physiology* **26:** pp. 1-88.

Williams, F.E., Varanasi, U. & Trumbly, R.J. (1991). The CYC8 and TUP1 proteins are involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. *Molecular and Cellular Biology* **11:** (6) pp. 3307-3316.

Xu, J. (2000). MAP kinases in fungal phytopathogens. *Fungal Genetics and Biology* **31:** pp. 137-152.

Yamashiro, C.T., Ebbole, D.J., Lee, B. Brown, R.E., Bourland, C., Madi, L. & Yanofsky, C. (1996). Characterization of *rco-1* of *Neurospora crassa*, a pleiotropic gene affecting growth and development that encodes a homolog of Tup1 of *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **16:** (11) pp. 6218-6228.

Yu, J. & Keller, N. (2005). Regulation of secondary metabolism in filamentous fungi. *Annual Review of Phytopathology* **43:** 437-458.

Zeilinger, S., Reithner, B., Scala, V., Piessl, I., Lorito, M. & Mach, R.L. (2005). Signal transduction by Tga3, a novel G protein α subunit of *Trichoderma atroviride*. *Applied and Environmental Microbiology* **71:** (3) pp. 1591-1597.

Zhang, Z. & Reese, J. (2004). Redundant mechanisms are used by Ssn6-Tup1 in repressing chromosomal gene transcription in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **279:** (38) pp. 39240-39250.

Zwiers, L.H. & De Waard M.A. (2001). Efficient *Agrobacterium tumefaciens*-mediated gene disruption in the phytopathogen *Mycosphaerella graminicola. Current Genetics* **39:** (5-6) pp. 388-393.

Chapter Seven
Appendices

7.1 Companies Cited in This Thesis

Advantec Toyo Kaisha, Ltd., Tokyo, Japan

Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK

Beckton Dickson & Co., Sparks MD, USA

Bio-Rad Laboratories Inc., Hercules CA, USA

Calbiochem, La Jolla, CA, USA

E-C Apparatus Corp., New York, NY, USA

Gentra Systems Inc., Minneapolis, MN, USA

Germantown NZ Ltd., Christchurch, New Zealand

GlaxoSmithKline Plc., Middlesex, UK

Invitrogen Corp., Carlsbad, CA, USA

Kodak (Australia) Pty., Ltd., Coburg, Vic., Australia

Lynnon Biosoft Corp., Quebec, Canada

Merck NZ Ltd, Palmerston North, New Zealand

Nalge Nunc International Inc., Naperville, IL, USA

NanoDrop Technologies Inc., Montchanin, DE, USA

Osram GmbH., Munich, Germany

Promega Corporation, Madison WI, USA

Qiagen Pty. Ltd., Clifton Hill, Vic., Australia

Roche Diagnostics GmbH., Mannheim, Germany

Sigma-Aldrich NZ Ltd., Auckland, New Zealand

Thermo Hybaid, Middlesex, UK

7.2 Methods

Unless otherwise stated, all chemicals are from Merck New Zealand Ltd.

7.2.1 Fungal Isolate Maintenance and Storage

Potato-dextrose Agar (PDA) (1 L)

39 g PDA (Beckton Dickson & Co.)

1 L H_2O

Autoclaved at 121° C for 15 min at 15 p.s.i.

Fungal isolates were cultured on PDA in Petri dishes at 22°C under 12 h light/dark cycles. For each isolate, 5 mm agar plugs from the actively growing colony margin were transferred to 10 mL PDA slopes in Universal bottles, incubated for 2 d under the same conditions as above, then sealed with parafilm and transferred to 4°C for storage.

7.2.2 Conidial Recovery for use as Inoculum

Conidia were generated in bulk and stored at -80°C in 20% glycerol until used. Isolates were grown on PDA (Appendix 7.2.1) at 25°C under constant light. To each plate, 10 mL sterile H_2O containing Tween 80 (two drops per 500 mL) was pipetted and a glass rod used to scrape the conidia into suspension. Mycelium was removed by filtering the conidial suspension through sterile Miracloth (Calbiochem) and conidia were pelleted by centrifugation at 1000 x*g* for 2 min at 4°C, resuspended in 20% glycerol and stored at -80°C.

7.2.3 pH buffers

McIlvaine Buffers (McIlvaine, 1921)

Solution A 0.1 M Citric acid

Solution B 0.2 M Na_2HPO_4

For 1 L at 2 X concentration mix solutions A and B according to Table 6.1 below

Gomori Buffers (Sambrook *et al*, 1989)

Solution C 1 M K_2HPO_4

Solution D 1 M KH_2PO_4

For 1 L at 2 X concentration mix solutions C and D according to Table 6.2 below and then add 800 mL H_20

Table 7.1. McIlvaine Buffers

	mL	
pH	**A**	**B**
2.8	841.5	158.5
3.2	753	247
3.6	678	322
4.0	614.5	385.5
4.4	559	441
4.8	507	493
5.2	464	536
5.6	420	580

Table 7.2. Gomori Buffers

	mL	
pH	**C**	**D**
5.8	17	183
6.2	38.4	161.6
6.6	76.2	123.8
7.0	123	77
7.4	160.4	39.6
7.8	181.6	18.4

pH 4.0 unbuffered PDA (4PDA)

24 g PDB (Beckton Dickson & Co.)

15 g Agar (Germantown NZ Ltd., Christcurch, New Zealand)

380 µL 50% HCl

2 X 500 mL H20

Mixed PDB, HCl and 500 mL H_2O

Mixed agar and 500 mL H_2O

Autoclaved separately at 121° C for 15 min at 15 p.s.i.

Mixed immediately prior to pouring

7.2.4 Genomic DNA Extraction

Potato-dextrose Broth (PDB) (1 L)

24 g PDB (Beckton Dickson & Co.)

1 L H_2O

Autoclaved at 121° C for 15 min at 15 p.s.i.

Harvesting of Mycelium

Mycelial plugs (5 mm diameter) from the stored slope cultures were transferred to PDA in Petri dishes and incubated for 3 d at 22°C under 12 h light/dark cycles. A 5 mm agar plug from each actively growing colony margin was transferred to a deep Petri dish containing approximately 20 mL of PDB and incubated as above for 3 d. Mycelium was

harvested by pressing between Miracloth (Calbiochem) and paper towels to remove excess liquid, wrapping in aluminium foil and snap freezing in liquid nitrogen. All mycelial samples were stored at -80°C until required.

Phenol/Chloroform Extraction of DNA from Solution

To a DNA sample in a solvent resistant centrifuge tube, 1 volume Tris-buffered phenol (Invitrogen Corp.) and 1 volume chloroform was added and the solution mixed by gentle inversion. Tubes were centrifuged at 12 000 x*g*, the upper aqueous phase removed to a fresh centrifuge tube and the remaining liquid discarded. If the interphase was chalky, the procedure was repeated until it became clear. To extract residual phenol, 2 volumes chloroform was added, tubes gently inverted and centrifuged at 12 000 x*g*. The upper aqueous phase containing DNA and salts was removed to a fresh tube and the remaining liquid discarded.

7.2.5 Agarose Gel Electrophoresis

50X TAE Buffer (Sambrook *et al.*, 1989) (1 L)

242 g	Tris base (Invitrogen Corp.)
57.1 mL	Glacial acetic acid
100 mL	0.5 M EDTA (pH 8.0)

Made up to 1 L with H_2O

Autoclaved at 121°C for 15 min at 15 p.s.i.

6 X Loading Buffer (Sambrook *et al.*, 1989)

0.25 %	Bromophenol blue
0.25 %	Xylene cyanol FF
40 % (w/v)	Sucrose in water

500 µL aliquots stored at 4°C

1% Agarose Gel (Sambrook *et al.*, 1989)

A 1% agarose gel was prepared by dissolving 1 g of agarose (Agarose MP, Roche Diagnostics GmbH.) in 100 mL of 1 X TAE buffer by boiling. The dissolved agarose was cooled to 60°C, poured into a gel casting tray (10 cm x 6.5 cm) containing a well-forming comb and allowed to set for ~1 h. The comb was removed and the casting tray containing the solidified gel transferred to an electrophoresis tank (E-C Apparatus Corp.) containing 1 X TAE buffer.

DNA Quantification

To quantify genomic DNA, 2 µL aliquots mixed with 3 µL 6 X loading buffer and 13 µL water were loaded into individual wells of a 1% agarose gel. A 2 µL aliquot of DNA High Mass™ Ladder (Invitrogen Corp.) prepared in the same manner was loaded into an adjacent well. DNA was separated by electrophoresis at a constant electric voltage (12.5 V/cm) for ~50 min. The gel was stained by submerging in 0.5 µg mL^{-1} ethidium bromide for ~30 min. DNA was visualised on a transilluminator and a digital image captured using a Versadoc Imaging System Model 3000 (Bio-Rad Laboratories Inc.) DNA was quantified by visual comparison of sample DNA intensity with that of the DNA High Mass™ ladder.

PCR Product Size Determination

To estimate molecular weight of PCR products, 5 µL aliquots were mixed with 1 µL 6 X loading buffer and separated by agarose electrophoresis as described above. A 5 µL aliquot of a 1 kb Plus DNA Ladder™ (Invitrogen Corp.) was separated in an adjacent well. PCR product size was estimated by position in the gel relative to the known molecular weight bands in the ladder.

7.2.6 Gene Transformation Media

100 mg/mL Ampicillin Stock

500 mg Ampicillin (Roche Diagnostics GmbH.)

5 mL H_2O

Filter sterilised to 0.45 µm

500 µL aliquots stored at -20°C

25 mg/mL Kanamycin Stock

125 mg Kanamycin sulphate (Sigma-Aldrich Pty., Ltd.)

5 mL H_2O

Filter sterilised to 0.45 µm

500 µL aliquots stored at -20°C

25 mg/mL Rifampicin Stock

125 mg	Rifampicin (Sigma-Aldrich Pty., Ltd.)
5 mL	Dimethylsulfoxide (DMSO) (Sigma-Aldrich Pty. Ltd.)

Filter sterilised to 0.45 µm

500 µL aliquots stored at -20°C

100 mg/mL Timentin Stock

500 mg	Timentin (GlaxoSmithKline Plc., Middlesex, UK)
5 mL	H_2O

Filter sterilised to 0.45 µm

500 µL aliquots stored at -20°C

50 mg/mL X-gal Stock

Dissolved 100 mg of 5-bromo-4-chloro-3-indolyl-β-d-galactoside (Roche Molecular Biochemicals) in 2 mL N,N'-dimethyl-formamide (Sigma-aldrich Pty., Ltd.) in a sterile Universal and covered with aluminium foil to omit light. X-gal stock was stored at -20°C.

SOC Medium (Promega Corp.) (100 mL)

2 g	Bacto-tryptone (Beckton Dickson & Co.)
0.5 g	Yeast extract (Beckton Dickson & Co.)
100 mM	NaCl
400 mM	KCl
40 mM	$MgCl_2$ (filter-sterilised)
20 mM	Glucose (filter-sterilised)

The bacto-tryptone, yeast extract, NaCl and KCl were dissolved in 95 mL H_2O and autoclaved at 121°C for 15 min at 15 p.s.i.. The $MgCl_2$ and glucose were added and the total volume brought up to 100 mL with sterile H_2O. The medium was then filter sterilised to 0.2 µm and stored in 1 mL aliquots at -20°C until required

Luria Betini (LB) agar

10 g	Bacto-tryptone (Beckton Dickson & Co.)
5 g	Yeast extract (Beckton Dickson & Co.)
5 g	NaCl
15 g	Agar (Germantown NZ Ltd.)
1 L	H_2O

Autoclaved at 121°C for 15 min at p.s.i.

Agar was omitted to prepare LB broth

Yeast Mannitol (YM) agar

0.4 g	Yeast extract
10 g	Mannitol
0.1 g	NaCl
0.1 g	$MgSO_4$
0.5 g	$K_2HPO_4.3H_2O$
15 g	Agar (Germantown NZ Ltd.)
950 mL	H_2O

All ingredients except agar were dissolved in 950 mL H_2O

pH adjusted to 7.0 and volume made up to 1 L with H_2O

Autoclaved at 121°C for 15 min at p.s.i.

Agar was omitted to prepare the broth

Blue/White Selection Agar (Promega Corp.)

Ampicillin was added to LB agar, just prior to pouring, to give a final concentration of 100 µg mL^{-1}. Using a glass spreader, 20 µL of the 50 mg mL^{-1} X-gal stock was spread over the surface of dry LB/ampicillin plates and allowed to absorb for 10 min prior to use.

7.2.7 Nucleic Acid Hybridisation

20 x SSC (Sambrook *et al.*, 1989)

3 M	NaCl
0.3 M	*tri*-Sodium Citrate

Autoclaved at 121° C for 15 min at p.s.i..

10 x MOPS Buffer (Sambrook *et al.*, 1989)

41.2 g	MOPS
4.1 g	Sodium acetate (anhydrous)
3.7 g	EDTA

The MOPS, sodium acetate and EDTA were dissolved in 800 mL H_2O and the pH adjusted to 7.0 with NaOH. The total volume brought up to 1000 mL with H_2O the buffer was then filter sterilised to 0.2 µm and stored at room temperature protected from the light.

RNA Sample Buffer

1.5 mL	10 X MOPS
5.5 mL	formaldehyde
15 mL	formamide

500 µL aliquots stored at -20°C

RNA Loading Buffer

2.5 g	Ficoll400
40 mg	Bromophenol blue
40 mg	Xylene cyanol

Make up to 10 mL with H_2O

7.3 Conidiation Experiments

7.3.1 Effect of Constant Light on *T. hamatum* LU592 Grown on PDA at 25°C

T. hamatum LU592 grown under constant dark or constant light at 25°C. Plates were inoculuated with a conidial suspension and left unsealed (Figure 7.1).

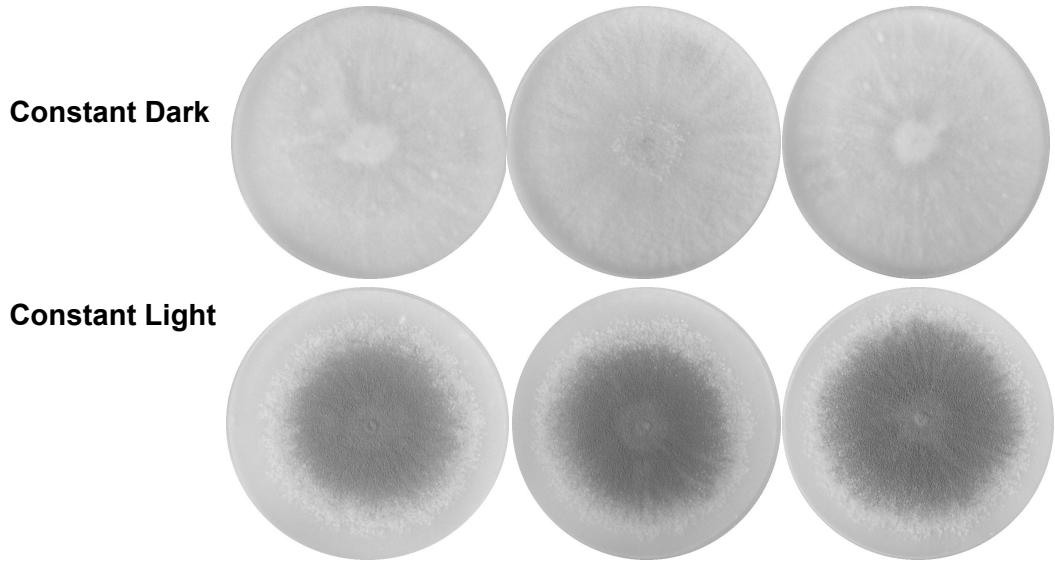


Figure 7.1. Effect of constant light on conidiation in *T. hamatum*. Culture were grown for 10 days on standard PDA at 25°C under either constant light or total darkness.

7.3.2 Effect of Media Volume on Photoconidiation in *T. atroviride*

In the PDB pH assays (2.15.4) there was liquid present in the *T. atroviride* plates at the end of the experiment, whereas in the first PDA experiment (2.15.1.2) and the PDYC pH assays (2.15.3) little or no free liquid was observed in the medium. It is possible the PDB plates contained more broth than the PDYC experiment, so this was tested as a variable using unbuffered PDB acidified to pH 4.4 and 4.8. Experimental design was as described in 2.14.4 except 2 or 3 mL PDB was added directly to the filter. Both the control and treatment plates were indistinguishable from the unbuffered PDB pH 4.4 and 4.8 treatments described earlier (Figure 7.2). In comparison with the 3 mL treatment plates conidiation appeared slightly more intense on 2 mL broth.

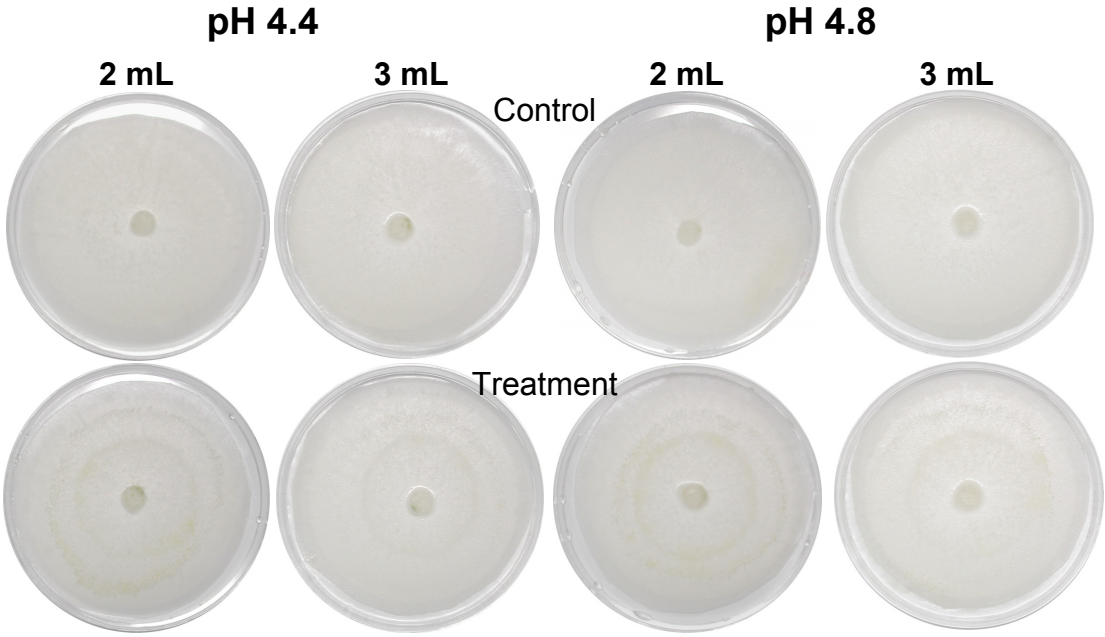


Figure 7.2. The effect of medium volume on photoconidiation in *T. atroviride* on unbuffered, pH adjusted (4.4 to 4.8) PDB-soaked filter paper. Filter papers received either 2 mL or 3 mL broth. Control = plates were grown for 4.75 d (114 h) in total darkness. Treatment = plates were grown for 42 h in total darkness, then photoinduced and grown a further 96 h in total darkness.

7.3.3 Effect of Light Exposure Timing on Photoconidiation in *T. atroviride*

In the pH filter paper assays exposure to blue-light was given at 42 h rather than 36 h. To assess whether this may have caused the variation in phenotype the effect of the timing of light exposure on photoconidiation was investigated using unbuffered PDB acidified to pH 4.4 and 4.8 and standard PDB. Experimental design was as described in 2.14.1 and 2.14.4 except 2 mL PDB was added directly to the filter and plates were exposed to light at 36 or 42 hours. In stark contrast to the previous assay (7.3.2) and the unbuffered PDB assays (2.15.3.2), two photoconidiation phenotypes were observed on the treatment plates. The two phenotypes occurred randomly and appeared to have no relationship to the timing of light treatment or the media (Figure 7.3). The first phenotype was a ring of profuse conidiation green in pigment as observed in the first PDB experiment (2.15.1.2). The second phenotype was the two immature rings of conidiation observed in the unbuffered PDB pH assays (2.15.4.1) and in the media volume experiment above (2.19.1). The single ring and the outer ring of the double correlated with the position of the colony margin at the time of light exposure.

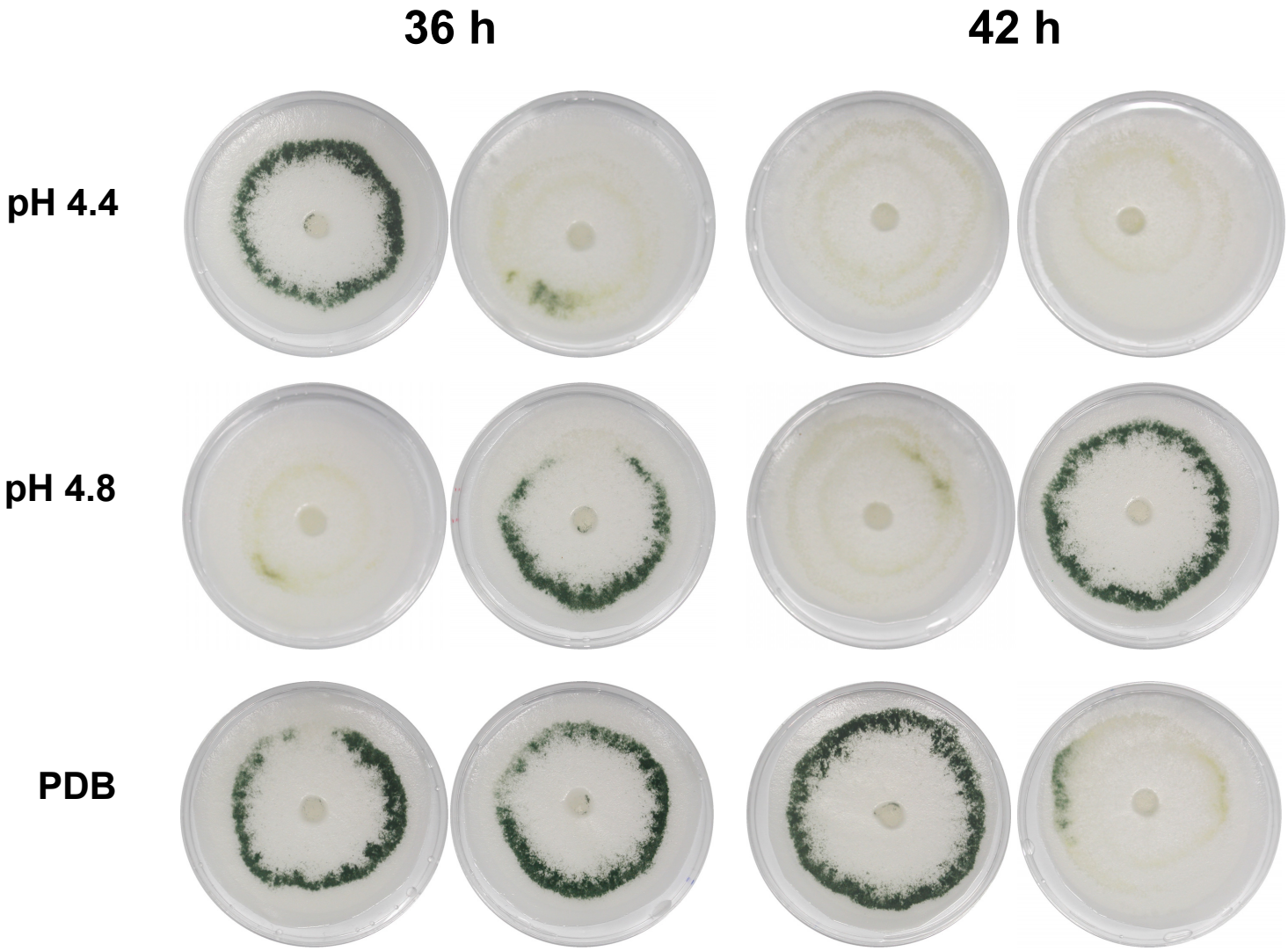


Figure 7.3. The effect of timing of light exposure on photoconidiation in *T. atroviride* on unbuffered, pH adjusted (4.4 to 4.8) PDB or standard PDB-soaked filter paper. Plates were grown for 36 h or 42 h in total darkness, then photoinduced and grown a further 72 h in total darkness. Both replicates are shown for each light exposure and media combination.

7.3.4 Preparation and Density of Filter Paper as a Source of Variation

Two different autoclave settings were used to sterilise the filter papers: a standard setting and a dry exhaust setting, in which the chamber is exhausted of moisture which leaves the contents dry. Filter papers in the dry exhaust run would have been wet then dried fully before adding the broth, whereas filters from the standard run were slightly moist when the broth was added. If this affected photoconidiation it may account for the two photoconidiation phenotypes observed in the previous section. The sterilisation method was tested as a possible source of variation. Filter paper density was also examined in this experiment. In the first assay filters were sterilised in an industrial autoclave on either the exhaust setting, which evacuates the chamber of moisture drying the chamber contents, or the standard setting, which leaves filters moist, or they were sterilised in a stove-top pressure cooker. Standard PDB was used as the medium and experiment design was as described in 2.14.1 except 2 mL PDB was added directly to the filter and plates were exposed to light at 42 hours. In the second assay different grades of filter paper were investigated in addition to the sterilisation method. Filter papers were sterilised using either the exhaust or standard setting described above and either one 70 mm Whatman #1 overlaid with an 80 mm Whatman #50, or two 70 mm Whatman #1 filter papers were placed in Petri dishes and 2 mL standard PDB was added to the filters as described above. Like the previous assay two photoconidiation phenotypes were observed on the treatment plates and the occurance of these appeared to have no relation to the sterilisation method treatments (Figure 7.4). In contrast, a single ring of profuse conidiation was observed on all the plates containing two Whatman #1 filters and this was also independent of sterilisation method, which suggested the source of the variation may the Whatman #50 filter papers (Figure 7.5).

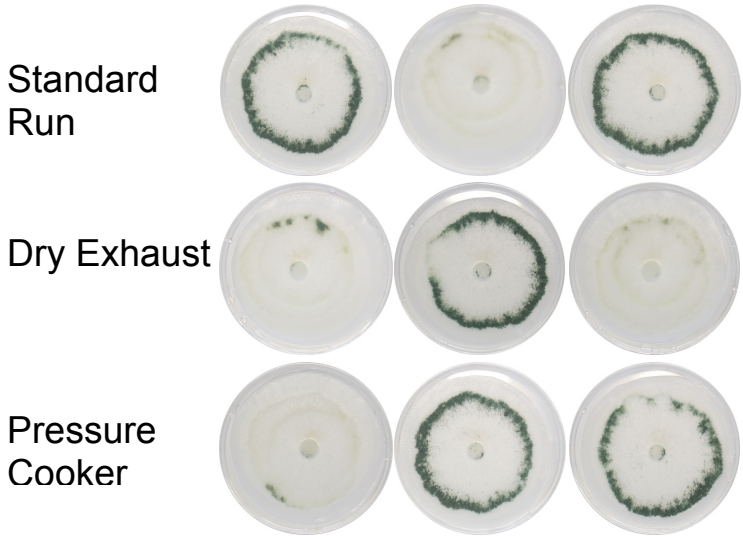


Figure 7.4. The effect of filter paper sterilization method on photoconidiation in *T. atroviride* on standard PDB-soaked filter paper.

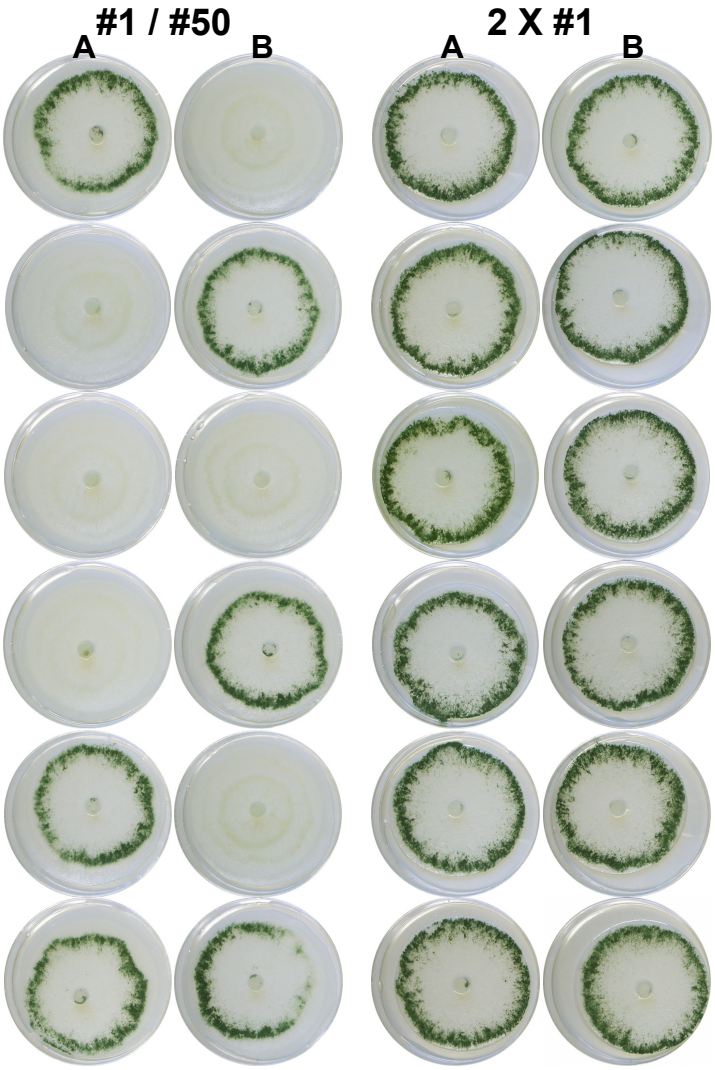


Figure 7.5. The effect of filter paper density (a Whatman #1 overlaid with a Whatman #50 [#1 / #50] or two Whatman #1 filters [2 X #1]) and sterilisation method (standard [A] versus dry exhaust [B]) on photoconidiation in *T. atroviride* on PDB-soaked filter paper.

7.3.5 Influence of Different Brands of Filter Paper

Two different brands of #50 density filter paper were available for our studies and while it was assumed Whatman #50 was used in all experiments, it is possible both Whatman #50 and Toyo Roshi No.4A (Advantec Toyo Kaisha, Ltd., Tokyo, Japan) were used at random. The effect of the different brands of filter paper on photoconidiation was investigated. Experimental design was as described in 2.14.1 except 2 mL PDB was added directly to the filters and plates were exposed to light at 42 hours and two Whatman #1, or one Whatman #1 overlaid with either one Whatman #50 or one Toyo Roshi No.4A were placed in Petri dishes. Half of the ten replicate plates containing a Whatman #1 overlaid with a Toyo Roshi No.4A produced a single green ring of conidia. In comparison only two of the treatments containing a Whatman #1 and Whatman #50 produced a green ring. In contrast to previous assays, a range of phenotypes from the two immature rings to an immature single ring to a green single ring were produced on the Whatman #1 / Whatman #50 plates. Immature single rings of conidiation were observed on the plates containing two Whatman #1 filter papers and, in addition, only two replicates produced a single green ring. This differed from the previous assay in which a single green ring was observed on all plates. These result suggested that different filter papers may ameliorate the variation effect, however they are not the cause. Examples of the different phenotypes and a summary of the proportion of plates most resembling each example is presented in Figure 7.6.

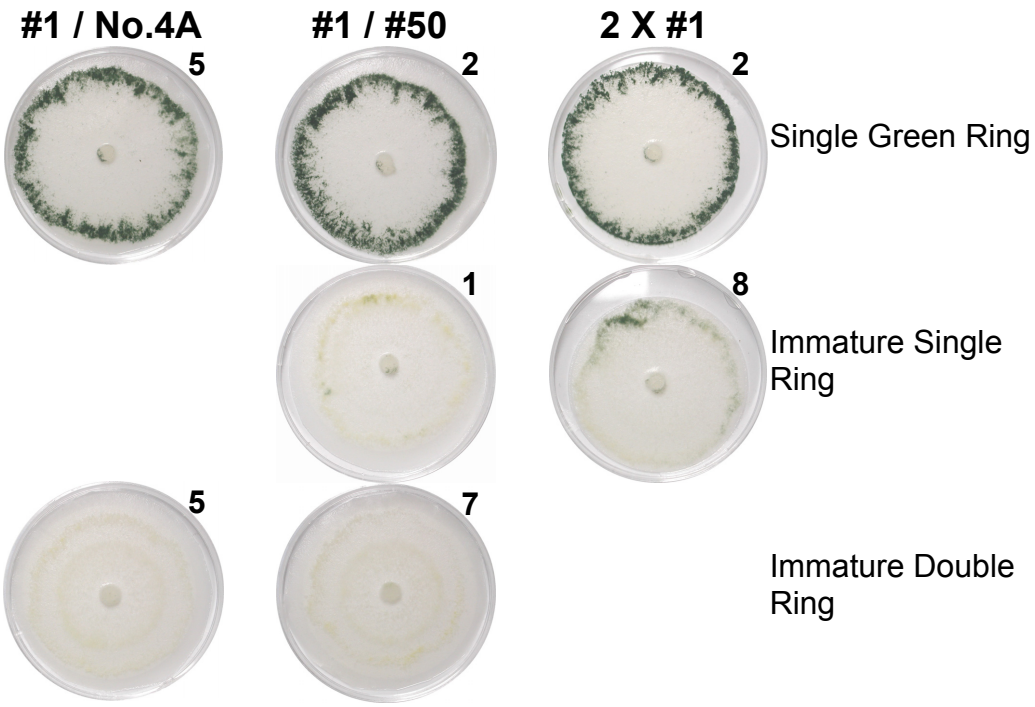


Figure 7.6. The effect of different filter paper brands and density (a Whatman #1 overlaid with either a Toyo Roshi No.4A [#1 / No.4A] or a Whatman #50 [#1 / No.4A], or two Whatman #1 filters only [2 X #1]) on photoconidiation in *T. atroviride.* The number of replicates from each treatment most resembling each example is indicated.

7.3.6 Effect of Inhibitory Compounds in Incubator

Studies have shown *Trichoderma* to produce volatile compounds which may be inhibitory to other organisms, such as 6-PAP production by *T. atroviride* (El-Hasan *et al.,* 2007). It was postulated that because of the high numbers of *T. atroviride* plates present in the incubator an auto-inhibitory substance was being produced which was the cause of the variation in comparison with the first experiment in which only four *T. atroviride* plates at a time were present in the incubator. This possibility was explored in a further set of assays. To assess whether an inhibitory compound was present in the incubator *T. atroviride* was grown in three different locations: in the original incubator, in a second incubator in another laboratory, in which *Trichoderma* spp. have never been grown, and in a third laboratory on the bench. Experimental design was as described in 2.14.1 except cultures were grown on a Whatman #1 overlaid with a Toyo Roshi No.4A and 2 mL PDB added directly to the filters in Petri dishes. Filters were exposed to light at 42 h. No location effect was observed in the experiment, which suggested an inhibitory substance was not building up in the incubator. Of the ten replicates incubated in the original incubator, nine plates produced a double immature ring and

one plate produced an immature single ring in response to light (data not shown). All plates grown in the second incubator produced a double ring and of those grown on the lab bench one produced a single green ring and the remaining a double immature ring.

7.3.7 Effect of Inhibitory Compounds Produced During the Experiment, Isolate-specific Variation and Circadian Rhythm

In this experiment three assays were prepared and inoculated at the same time and performed in a single incubator. To assess whether inhibitory compounds produced during the experiment contributed to variability in *T. atroviride* plates were incubated sealed and unsealed and photo-induced together. Filters (Whatman #1 overlaid with a Toyo Roshi No.4A) were placed in the Petri dishes and 2 mL standard PDB added directly to the filters. Plates were exposed to light at 42 h. In the second assay three isolates of *T. atroviride* (LU298, LU136 and LU140) were photo-induced to assess whether the LU298 phenotype is an isolate or species-specific response. Experimental design was as described above, except plates were unsealed. In the third assay photoconidiation was assessed in *T. atroviride* LU298 using large filters and Petri dishes to look for rhythmic production of conidial rings in addition to the two observed in other assays. Filter papers (a 120 mm Whatman #1 overlaid with a 120 mm Whatman #50) were placed in 150 mm diameter Petri dishes and 5 mL standard PDB added directly to the filters. Filters were inoculated as described in 2.14.1 and incubated unsealed at 23°C in total darkness for 48 or 72 h, then exposed to blue light for 15 min as described in 2.2.3 and incubated for a further 72 h in total darkness.

No effect on photoconidiation due to the sealing of the plates was observed. On the unsealed treatment plates cultures produced either a double immature ring, in which the outer ring was barely visible, or the inner ring only. This differed from previous assays in which the outer ring which represented the colony front at the time of light exposure was always visible and demonstrates a further range of phenotypic responses. On the unsealed an immature double ring, described in previous assays, was produced in addition to the faint outer ring and inner ring only phenotype. No single green rings of conidia were formed on any plate. Examples of the photoconidiation phenotypes and a summary of the proportion of plates most resembling each example is presented in Figure 7.7.

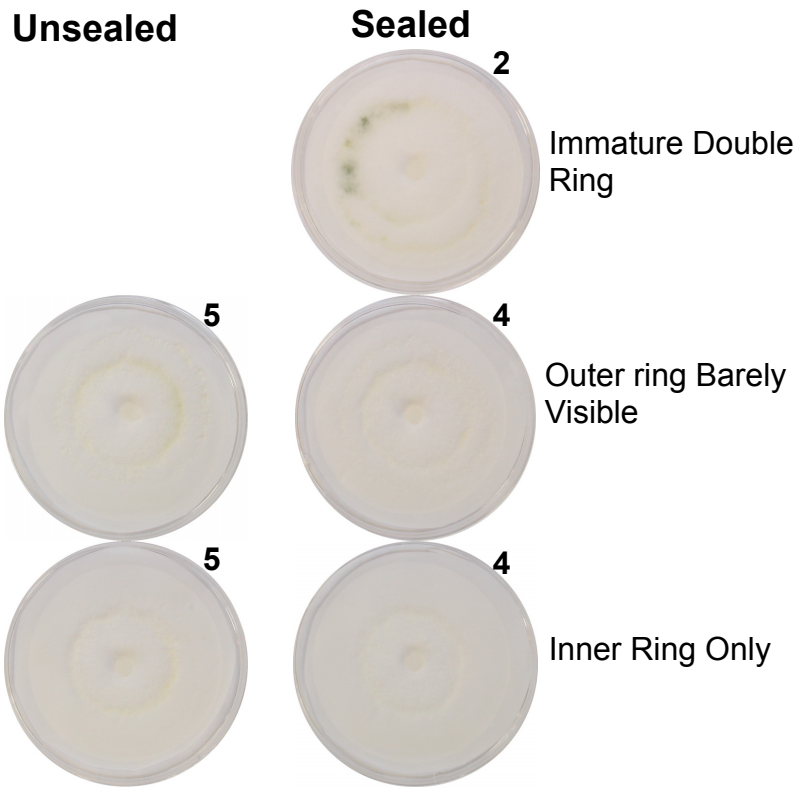


Figure 7.7. The effect of sealing the plates on photoconidiation in *T. atroviride.* The number of replicates from each treatment most resembling each example is indicated.

To test whether the altered photoconidiation phenotype observed in *T. atroviride* LU298 is an isolate specific phenomenon, two other *T. atroviride* isolates, in addition to LU298, were subjected to a photoconidiation assay on PDB-soaked filter paper. Of the five LU298 replicate plates, two produced a double immature ring in which the outer ring was barely visible and the remainder produced an inner ring only (Figure 7.8). Both of these phenotypes were observed in the previous assay. All of the five LU140 plates produced a single immature ring only, and this did not correlate with the colony margin at the time of light exposure. This ring was identical to the inner ring of the LU298 plates and was therefore assumed to be a variation on the double ring phenotype. A double immature ring of conidiation was barely visible on one of the LU136 plates and no conidiation was observed on the remaining four. In preliminary assays at the commencement of this study both of these isolates produced a single green ring of conidia in response to light (data not shown), which suggested the double ring phenotype was not due to isolate variation rather an unknown variable which affects *T. atroviride*.

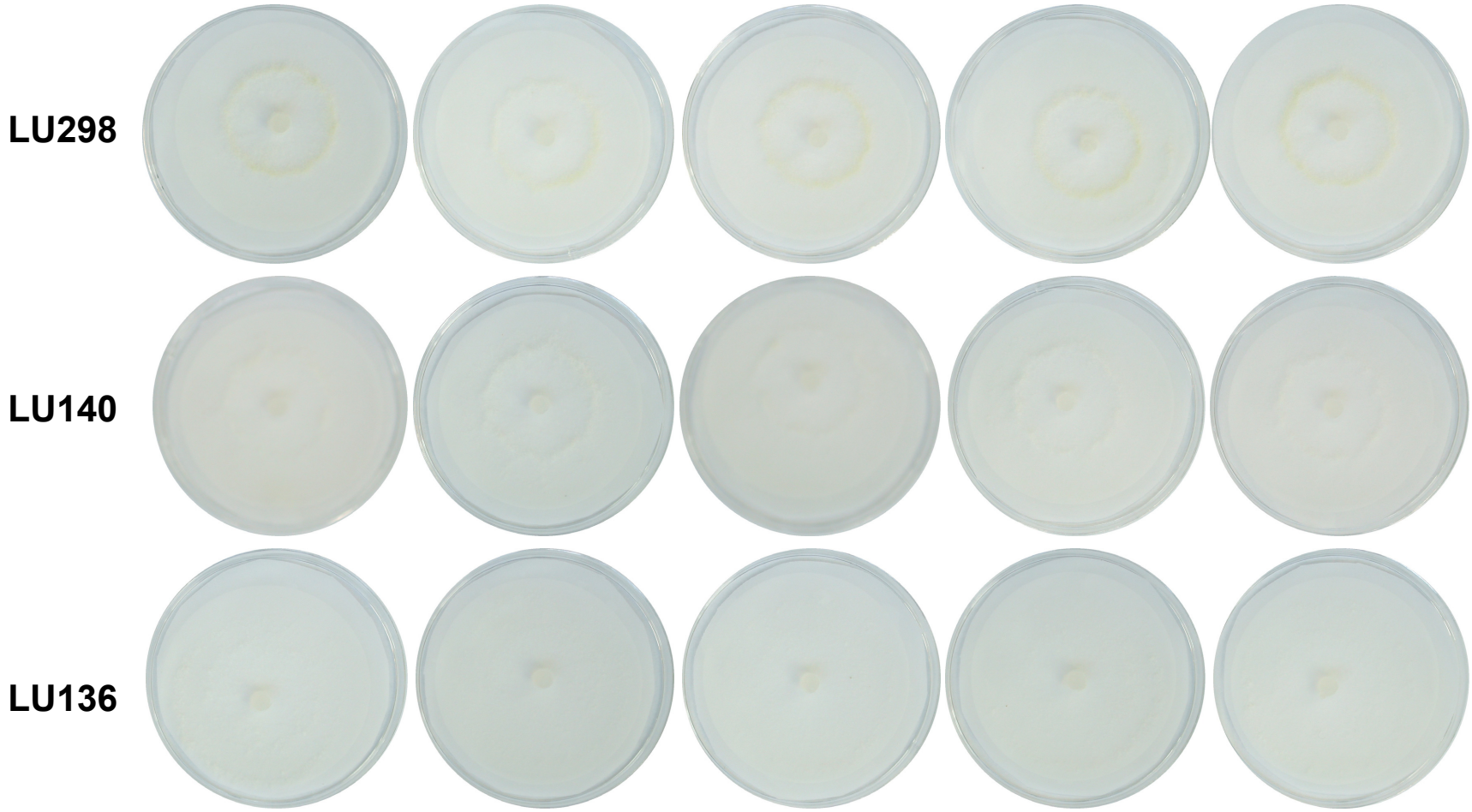


Figure 7.8. Photoconidiation assay on three isolates (LU298, LU140 and LU136) of *T. atroviride* on PDB-soaked filter paper. Plates were grown for 42 h in total darkness, then photoinduced and grown a further 72 h in total darkness. All five replicates for each isolate are presented.

It was postulated that the inner ring was initiated rhythmically by light exposure to the mycelial plug during preparation of the experiment and that if a single blue-light exposure was given at 72 h then two inner rhythmic rings and an outer light-induced ring should be produced. To test this hypothesis *T. atroviride* LU298 was subjected to a photoconidiation assay on 120 mm filter papers in 150 mm Petri dishes with light exposure at 48 h and 72 h. In stark contrast to the previous assays a single green ring of conidia was formed on all plates and the location of the ring correlated with the position of the colony front at the time of light exposure. As the double ring phenotype did not occur, it was not possible to determine if the inner ring was produced rhythmically.

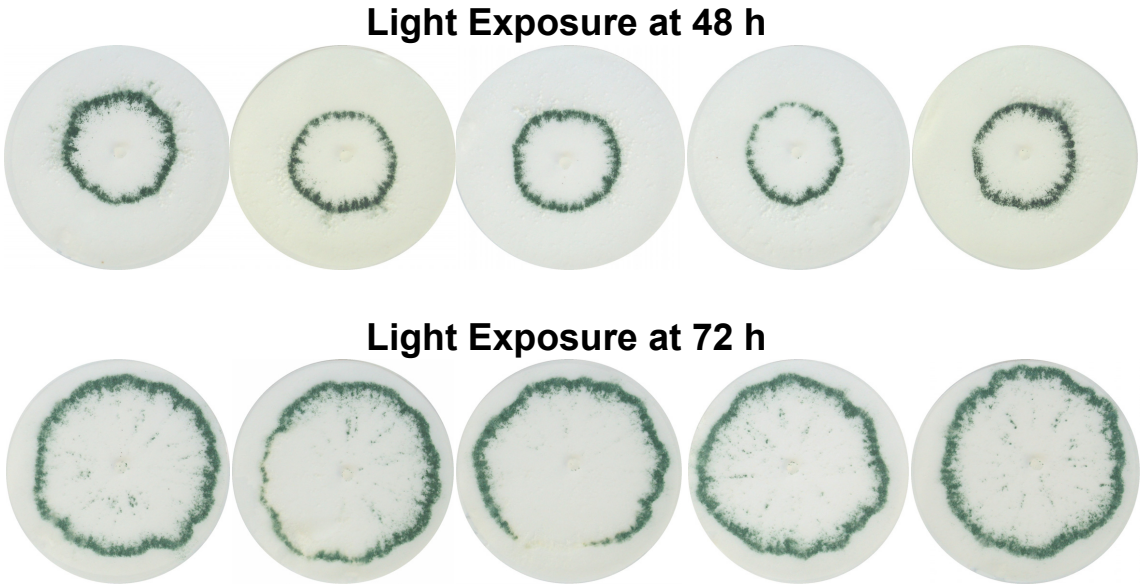


Figure 7.9. *Trichoderma atroviride* photoconidiation assay on larger size PDB-soaked filter papers. Cultures were grown for 48 h or 72 h in total darkness, then photoinduced and grown a further 72 h in total darkness. All five replicates are presented.

7.4 Sequences Isolated in this Study

7.4.1 *Trichoderma* spp. *blr-1*

7.4.1.1 *Trichoderma* spp. *blr-1* Nucleotide Sequence Alignment

Partial alignment of the *blr-1* nucleotide sequences isolated in this study from *T. atroviride* LU298, *T. hamatum* LU592 and *T. harzianum* LU675. The isolated region is situated approximately 1040 bp 3' to the predicted translational start site. Consensus bases are in blue. Numbering is relative to this region only.

```
LU298      tcgtctgtgatgtcacgatgaatgattgtcccattatttacgtatctgataatttccaga     60
LU592      tcgtttgtgatgtcacgatgaatgattgtcccattatatacgtatctgataatttccaga     60
LU675      tcgtttgtgatgtcacgatgaatgattgccctataatttatgtatccgataatttccaga     60
           tcgt tgtgatgtcacgatgaatgattg cc at at ta gtatc gataatttccaga

LU298      acctcactggatacagccgccatgaaatcattggacaaaactgccgcttccttcaagcac    120
LU592      acctcactggatacagccgccatgaaattattggacaaaattgtcgcttccttcaagcac    120
LU675      atctaactgggtacagccgccatgagatcatcggacaaaactgtcgcttccttcaagcgc    120
           a ct actgg tacagccgccatga at at ggacaaaa tg cgcttccttcaagc c

LU298      ccgatggaaaggtggaagcaggatcgaagcgagagtttgtggacgacggggccgtgttta    180
LU592      ccgatggaaaggttgaagcagggtcaaagcgagagtttgtggacgacggagccgtgttta    180
LU675      ccgatgggaaagtcgaggccggttcaaaacgagaatttgttgatgacggagcagtgttta    180
           ccgatgg aa gt ga gc gg tc aa cgaga tttgt ga gacgg gc gtgttta

LU298      acctcaaaagaatgataaaggagggtcgcgaagtgcaacagagcttgataaactaccgca    240
LU592      acctcaaaagaatgataaaagagggccgcgaagtacaacagagcttaatcaactaccgca    240
LU675      atctcaaacggatgatccaggagggccgtgaggtgcagcaaagcttaatcaactatcgaa    240
           a ctcaaa g atgat  a gaggg cg ga gt ca ca agctt at aacta cg a

LU298      agggaggaaagccctttctgaacctcttgacgatgatacctataccctgggacactgatg    300
LU592      aaggaggaaagcccttcctcaatctcttgacgatgatacctattccctgggatactgatg    300
LU675      agggaggaaaacctttcctcaacctcttgaccatgataccaataccatgggataccgatg    300
           a ggaggaaa cc tt ct aa ctcttgac atgatacc at cc tggga ac gatg

LU298      aaataaggtacttcatcggctttcagattgatcttgttgaatgccctgacgccatctctg    360
LU592      aaataagatacttcatcggctttcagatcgatcttgttgaatgccccgacgccatctctg    360
LU675      agatcagatactttattggcttccaaattgaccttgtcgaatgccccgacgccatctctg    360
           a at ag tactt at ggctt ca at ga cttgt gaatgccc gacgccatctctg

LU298      gtcaagaaatgggaggcgtcaaggtgaactacaaacacaacgatattgggcagtatatat    420
LU592      gtcaagaaatgggaggcgtcaaggtgaactataaacacagcgatattggccaatatatat    420
LU675      gccaggaactaggcggcgtcacagtcaactataaacacagcgacattggccaatatatat    420
           g ca gaa t gg ggcgtca  gt aacta aaacaca cga attgg ca tatatat

LU298      ggactccaccaccatctaatcctttggaatccgagaacggtcagacactcggggtggatg    480
LU592      ggaccccaccgccatctaatcatttggaacccgagaacggccagacgcttggagtggatg    480
LU675      ggactccgccggtgccaaaccagttggagtctgacaatggacagacgctcggtgtggatg    480
           ggac cc cc     c aa c  ttgga  c ga aa gg cagac ct gg gtggatg

LU298      atgtttctaccctccttcagcagtataatccgagcgggctcgtttcggattggcataaac    540
LU592      atgtttctaccctccttcagcagtataatccgaacgggctcgtttcggattggcataaac    540
LU675      atgtctctacactcctacagcagtacaatccgaacggactcgtatcagattggcataaat    540
           atgt tctac ctcct cagcagta aatccga cgg ctcgt tc gattggcataaa

LU298      aatcgtgggataagatgctcctcgaaaatgccgacgacgtggtccatgttatatcactta    600
LU592      agtcatgggataagatgcttctcgaaaatgctgacgatgtggtccacgttatatcactta    600
LU675      cgtcctgggacaagatgcttctcgagaatactgatgatgtggtccacgttatatcactca    600
             tc tggga aagatgct ctcga aat c ga ga gtggtcca gttatatcact a

LU298      aaggactcttcctttacctatcaccttcctgcaaacgtattctcgaatacgatgccgctg    660
LU592      aaggactcttcctttacctgtcgccatcttgcaagcgcattctcgaatatgatgctgctg    660
LU675      agggcctctttctctatctatcgccttcctgtaagcgtgttctcgagtacgatggcgccg    660
           a gg ctctt ct ta ct tc cc tc tg aa cg  ttctcga ta gatg  gc g
```

```
LU298       acctggttgggaactctttatcctccgtttgtcatccttcagacattgttcccgtcacgc    720
LU592       acctggttggaaactccttgtcttccgtttgccacccttcagacattgttcccgtcacac    720
LU675       acctagttggtaacccctttgtcctcggtttgccacccttcggacattgttccagtgacgc    720
            acct gttgg aac c tt tc tc gtttg ca ccttc gacattgttcc gt ac c
```

7.4.1.2 *Trichoderma* spp. BLR-1 Amino Acid Alignment

Partial alignment of the BLR-1 amino acid sequences from *T. atroviride* LU298, *T. hamatum* LU592 and *T. harzianum* LU675. The predicted amino acid sequences were derived using Wise2 (http://www.ebi.ac.uk/Wise2). The sequence begins approximately at protein position 190. Consensus amino acids are in red. Numbering is relative to this region only.

```
LU298       VCDVTMNDCPIIYVSDNFQNLTGYSRHEIIGQNCRFLQAPDGKVEAGSKREFVDDGAVFN     60
LU592       VCDVTMNDCPIIYVSDNFQNLTGYSRHEIIGQNCRFLQAPDGKVEAGSKREFVDDGAVFN     60
LU675       VCDVTMNDCPIIYVSDNFQNLTGYSRHEIIGQNCRFLQAPDGKVEAGSKREFVDDGAVFN     60
            VCDVTMNDCPIIYVSDNFQNLTGYSRHEIIGQNCRFLQAPDGKVEAGSKREFVDDGAVFN

LU298       LKRMIKEGREVQQSLINYRKGGKPFLNLLTMIPIPWDTDEIRYFIGFQIDLVECPDAISG    120
LU592       LKRMIKEGREVQQSLINYRKGGKPFLNLLTMIPIPWDTDEIRYFIGFQIDLVECPDAISG    120
LU675       LKRMIQEGREVQQSLINYRKGGKPFLNLLTMIPIPWDTDEIRYFIGFQIDLVECPDAISG    120
            LKRMI EGREVQQSLINYRKGGKPFLNLLTMIPIPWDTDEIRYFIGFQIDLVECPDAISG

LU298       QEMGGVKVNYKHNDIGQYIWTPPPSNPLESENGQTLGVDDVSTLLQQYNPSGLVSDWHKQ    180
LU592       QEMGGVKVNYKHSDIGQYIWTPPPSNHLEPENGQTLGVDDVSTLLQQYNPNGLVSDWHKQ    180
LU675       QELGGVTVNYKHSDIGQYIWTPPVPNQLESDNGQTLGVDDVSTLLQQYNPNGLVSDWHKS    180
            QE GGV VNYKH DIGQYIWTPP  N LE  NGQTLGVDDVSTLLQQYNP GLVSDWHK

LU298       SWDKMLLENADDVVHVISLKGLFLYLSPSCKRILEYDAADLVGNSLSSVCHPSDIVPVT     239
LU592       SWDKMLLENADDVVHVISLKGLFLYLSPSCKRILEYDAADLVGNSLSSVCHPSDIVPVT     239
LU675       SWDKMLLENTDDVVHVISLKGLFLYLSPSCKRVLEYDGADLVGNPLSSVCHPSDIVPVT     239
            SWDKMLLEN DDVVHVISLKGLFLYLSPSCKR LEYD ADLVGN LSSVCHPSDIVPVT
```

7.4.2 *Trichoderma* spp. *frq*

7.4.2.1 *Trichoderma* spp. *frq* Nucleotide Sequence Alignment

Partial alignment of the putative *frq* nucleotide sequences isolated in this study from *T. atroviride* LU298, *T. hamatum* LU592 and *T. harzianum* LU675. The isolated region is situated approximately 630 bp 3' to the predicted translational start site. Consensus bases are in blue. Numbering is relative to this region only.

```
LU298       gacactccgagactttacagcaagcttaggagattcaccgaacgccacttcatcgaagcg     60
LU592       gacgctccgagaatttacggcaagcttaggagactcgccagatacctcttcattgaagcg     60
LU675       cacgctccgagacttcacagcgactttgggagactcgcccgacacctcttcgtctcagcg     60
             ac ctccgaga tt ac gc a  tt ggaga tc cc  a  cc cttc t  agcg

LU298       cgacaa...gaaatcgtccaggcatgctactcgcgacgggatgtattcaggatcaggctc    117
LU592       cgacaa...aaaatcgtccaggcatgctactcgcgatgggatgtattctggatcaggctc    117
LU675       taacaataaaaaatcatcgaggcatgccacgcgcgatcggatgtattctggttcgggctc    120
              acaa    aaatc tc aggcatgc ac cgcga  ggatgtattc gg tc ggctc

LU298       catgtcattatcgaaacacgcctcgtcttcgtctggatctcacacgcggccagttgactc    177
LU592       catgtcactatcgaagcatgcctcgtcttcgtctggatctcacacacggccagttgactc    177
LU675       aaagtcacagtcgaagcatgcttcgtcgtcatctggatcgcatacacgaccagttgactc    180
             a gtca   tcgaa ca gc tcgtc tc tctggatc ca ac cg ccagttgactc
```

```
LU298      ggcttatgcgtccatgtcgattggtgccggctcttctggaacatcgttgaacaggccaca    237
LU592      ggcttatgcgtctatgtcgactggtgccggctcatctggaacatccttgaataggccaca    237
LU675      tgcctacgcgtccatgtcgactggtgctggctcgtcgggaacctcgttgcaccgacctca    240
            gc ta gcgtc atgtcga tggtgc ggctc tc ggaac tc ttg a  g cc ca

LU298      aggaggcgcgcgattcaagtctaatgaagcaaaagtagagaattacttgcgcgatatccc    297
LU592      agggggctcacggttcaagactaatgagcagaaagtagagaactacttgcgcgatatccc    297
LU675      aggaggctcacggttcaagacgaacgagcagaaagtagaaaactacttgcgcgagatccc    300
           agg ggc c cg ttcaag c aa ga    aaagtaga aa tacttgcgcga atccc

LU298      cgaggggctgtatccccgttctctgtccatgacggaaaaggaaaagaagaagcttgtggt    357
LU592      cgaggggctgtacccccgttctctctccatgacagaaaaggaaaagaggaagcttgtggt    357
LU675      cgaggggctgtatccccgttttgtcgccatgacggaaaaggaaaagaagaaactagtggt    360
           cgaggggctgta ccccgtt t t  ccatgac gaaaaggaaaaga gaa ct gtggt

LU298      ccgacgtcttgagcagatcttcacaggaaaatttacaggccggcatgcccgtcggaacca    417
LU592      ccgacgtcttgagcagctattcacaggaaaatttgcaggccggcatgctcgtcggaacca    417
LU675      ccggcgtcttgagcaaatcttcacggggaaaatcggaggcaagcatgttcgtcggagcca    420
           ccg cgtcttgagca  t ttcac gg aaa t   aggc  gcatg  cgtcgga cca

LU298      gcctagtcaaacggcacctgcatctactgcgcaagccccgttagggccgccaggagtaca    477
LU592      gcctaaacaaacggcagccgcatctaatacgcaggcccctgtggggcagtcaggagtgca    477
LU675      gcctcttcgaccgcctcccatcatcccaccgcag....cacaggcgatatcgggcaatcc    476
           gcct   c a cg c  c          cgca    c    g g    c gg    c

LU298      ctcttccctgcggccttcctctgttcagcagccgaaactaaagccaa......gtccaat    531
LU592      ctccccttgcgtcctttcactgttcagcagccgaaaccaaaaccaa......gtctaaa    531
LU675      ttctccccagcggtcctctctggtacagcagccaaagccaggcctgactgccaatcccag    536
            tc  cc  gcg  c t     gt cagcagcc aa c a   c  a       tc  a

LU298      tgccgaatctga...........gctcctgcgcgaagctcgaatacttccccaggaactt    580
LU592      tgttatttctga...........gcccctgcgtgaagctcgaattcttccccaggaac..    578
LU675      cgtcaactccaatgctgtaccaaaacccatgcgcgaagctcgaattcttccccaggaacat    596
            g     tc  a           c c tgcg gaagctcgaat cttccccaggaac

LU298      c......accacgcaggaaagaagagcagatcgcaagacataggctccgcgtccaactca    634
LU592      .......atcatgcaggaaagaagagcagatcacaagatatcggctctgcgtccaactcc    631
LU675      caatccggacatacgggaaaaaagagcagatcccaagacatgggctcagcgtccaattct    656
                    ca  c ggaaa aagagcagatc caaga at ggctc gcgtccaa tc

LU298      aacagagatcagacagaatcaggggtaacggcaatggcaatggtagtagca........    686
LU592      aacgaagatcagacagaatcaggggtaacggcaatggcagcggtt..............    677
LU675      aacgaagatcaaaccgaatcaggaggcaacggcaacagtagtggcaacagcaacggcaat    716
           aac  agatca ac gaatcagg gg aacggcaa  g a  gg

LU298      ....caggatcaaacccgtctccgcccatgccaccacctcctgaacagcgacccacaaga    742
LU592      ....caggatcaaacccatctccgcccatgccaccacctcctgaacagcgacccacgaga    733
LU675      ggctcagggacgaatacgtcgccgcctgtgccgccgcctcccgagcagcgacccacgagg    776
               cagg  c aa  c tc ccgcc  tgcc cc cctcc ga cagcgacccac ag
LU298      cccaaagacctcgatcctgaccgcatccaaatcccatccgaaaacatggagtacataaga    802
LU592      cccaaagatctagatccagatcgcattcaaatcccgtctgaaaacatggaatacataagg    793
LU675      cccttggatctcgatcctgatcgcgtccagatcccgtccgagaatatggagtacatacgg    836
           ccc   ga ct gatcc ga cgc t ca atccc tc ga aa atgga tacata g

LU298      catttaggtcttgtccctcctgagctgatgaccgatccacca......tcagaaattgac    856
LU592      catctaggtcttgtccctccagagctgatacccgatccgccagagcgctcagataacgat    853
LU675      catttaggtcttgttcctccagagctactctcaacccctccagaacggccaaacgacgag    896
           cat taggtcttgt cctcc gagct  t  c    cc cca       ca a    ga

LU298      gtccaccccgatgccgaagggtgggtttatttg                               889
LU592      gttcaccctgatgccgaggggtgggtttattta                               886
LU675      gttcatcctgacgaggagggctgggtgtatttg                               929
           gt ca cc ga g  ga gg tgggt tattt
```

7.4.2.2 *Trichoderma* spp. FRQ Amino Acid Alignment

Partial alignment of the FRQ amino acid sequences from *T. atroviride* LU298, *T. hamatum* LU592 and *T. harzianum* LU675. The predicted amino acid sequences were derived using Wise2 (http://www.ebi.ac.uk/Wise2). The sequence begins approximately at protein position 190. Consensus amino acids are in red. Numbering is relative to this region only.

```
LU298      TLRDFTASLGDSPNATSSKRD.KKSSRHATRDGMYSGSGSMSLSKHASSSSGSHTRPVDS     59
LU592      TLREFTASLGDSPDTSSLKRD.KKSSRHATRDGMYSGSGSMSLSKHASSSSGSHTRPVDS     59
LU675      TLRDFTATLGDSPDTSSSQRNNKKSSRHATRDRMYSGSGSKSQSKHASSSSGSHTRPVDS     60
           TLR FTA LGDSP   S  R  KKSSRHATRD MYSGSGS S SKHASSSSGSHTRPVDS

LU298      AYASMSIGAGSSGTSLNRPQGGARFKSNEAKVENYLRDIPEGLYPRSLSMTEKEKKKLVV    119
LU592      AYASMSTGAGSSGTSLNRPQGGSRFKTNEQKVENYLRDIPEGLYPRSLSMTEKEKRKLVV    119
LU675      AYASMSTGAGSSGTSLHRPQGGSRFKTNEQKVENYLREIPEGLYPRFVAMTEKEKKKLVV    120
           AYASMS GAGSSGTSL RPQGG RFK NE KVENYLR IPEGLYPR   MTEKEK KLVV

LU298      RRLEQIFTGKFTGRHARRNQPSQTAPASTAQAPLGPPGVHSSLRPSSVQQPKL....KPS    175
LU592      RRLEQLFTGKFAGRHARRNQPKQTAAASNTQAPVGQSGVHSPLRPFTVQQPKP....KPS    175
LU675      RRLEQIFTGKIGGKHVRRSQPS.TASHHPTAAQAIS.GNPSPQRSSLVQQPKPGLTANPS    178
           RRLEQ FTGK  G H RR QP  TA      A     G  S  R   VQQPK      PS

LU298      P.IAESELLREARILPQELHHAG..KKSRSQDIGSASNSNRDQTESGGNGNGNGSST...    229
LU592      L.NVISEPLREARILPQE.HHAG..KKSRSQDIGSASNSNEDQTESGGNGNGSGS.....    226
LU675      VNSNAVKPMREARILPQEHQSGHTGKKSRSQDMGSASNSNEDQTESGGNGNSSGNSNGNG    238
                     REARILPQE       KKSRSQD GSASNSN DQTESGGNGN  G

LU298      .GSNPSPPMPPPPEQRPTRPKDLDPDRIQIPSENMEYIRHLGLVPPELMTDPP..SEIDV    286
LU592      .GSNPSPPMPPPPEQRPTRPKDLDPDRIQIPSENMEYIRHLGLVPPELIPDPPERSDNDV    285
LU675      SGTNTSPPVPPPPEQRPTRPLDLDPDRVQIPSENMEYIRHLGLVPPELLSTPPERPNDEV    298
            G N SPP PPPPEQRPTRP DLDPDR QIPSENMEYIRHLGLVPPEL   PP      V

LU298      HPDAEGWVYL                                                      296
LU592      HPDAEGWVYL                                                      295
LU675      HPDEEGWVYL                                                      308
           HPD EGWVYL
```

7.4.3 *Trichoderma* spp. *rcoT* Nucleotide Sequence Alignment

Coding region alignment of the putative *rcoT* nucleotide sequences isolated in this study from *T. atroviride* LU298 and *T. hamatum* LU592. Consensus bases are in blue. Numbering is relative to the ATG start codon at position 1.

```
LU298      atgtccatgtactcgcatcgcggcatgggggctgtgcctcccggcaatgctcgcctgaac     60
LU592      atgtccatgtactcgcatcgcggcatgggggctgtgcctcccggcaatgctcgcctgaat     60
           atgtccatgtactcgcatcgcggcatgggggctgtgcctcccggcaatgctcgcctgaa

LU298      gagctgctcgagcagattagagccgagttcgacagccaacagcgtcagaccgagagcttt    120
LU592      gagctgctggagcaggtcagagctgagttcgacagccaacagcgtcaaaccgagagcttt    120
           gagctgct gagcag t agagc gagttcgacagccaacagcgtca accgagagcttt

LU298      gaacatcagagtacgcctcccttgcaaatactgtccataccgacaactattgcgcgcgtt    180
LU592      gagcatcagagtacgcttcccttgcatatgctatccataccgacgactattgtgcgcgtt    180
           ga catcagagtacgc tcccttgca at ct tccataccgac actattg gcgcgtt

LU298      tgattggggtggaatgtttttgagcaaagagcagaaaatttcgatttttctcatttcta    240
LU592      tgattgtggtggagggcttttgagaacaaaagagcaaaatttggaaaaaaaaaaaaagga    240
           tgattg ggtgga  g ttttgag a a a  ag aaa tt g          a     a
```

```
LU298      aaaacaa.........tcaaaaggcgttgcagtttgaagcaa.................    273
LU592      aaagaagagaaaaaaattcaaaaggcacaggagttgaaacaaagaaacatgagctgaaac    300
           aaa  a           tcaaaaggc   g agtt  aa  aa

LU298      .aagtatcatgagctaacctctcttcttcgatttatagtctcggcgcaagttagcgaaat    332
LU592      aaagaaacatgagctaatctgctttatttgatttatagtttcagcacaagttagcgaaat    360
            aag a catgagctaa ct   tt tt gatttatagt tc gc caagttagcgaaat

LU298      gcagctggtacgtgaaaaggtctatgccatggagcaaacgcatatgacgctcaagcaaaa    392
LU592      gcagctggtacgcgaaaaagtctatgccatggaacaaacgcatatgacgctcaagcaaaa    420
           gcagctggtacg gaaaa gtctatgccatgga caaacgcatatgacgctcaagcaaaa

LU298      gtaagc.....ttctcc....tcctacagcccgcacgcctcacatgtcgctaaccgcatc    443
LU592      gtaagcagcaactctcccgcatactgcaacccgcgcctctcacatgtcgctaatcacgtt    480
           gtaagc      tctcc    t ct ca cccgc c  ctcacatgtcgctaa c c t

LU298      tcaactctccagatatgaggaggaaatcaacatgctgcggcaccagctcgaacttgctcg    503
LU592      cttactccccagatatgaggaagagatcaacatgctgcggcatcagctcgaacttgctcg    540
              actc ccagatatgagga ga atcaacatgctgcggca cagctcgaacttgctcg

LU298      caagggagctccgcagtctggcctgcaagggcctcctcaacacgcagggccgtctcagca    563
LU592      caagggagctccgcagtcgggtctgcaaggacctcctcagcacgcagggccatctcagca    600
           caagggagctccgcagtc gg ctgcaagg cctcctca cacgcagggcc tctcagca

LU298      acccccatcgattgctcctggaaacggcctcttcagcggcatcatggccggcggcaacca    623
LU592      acccccatcgattgctcctggaaacggcctcttcagcggcatcatggccggtggcaacca    660
           acccccatcgattgctcctggaaacggcctcttcagcggcatcatggccgg ggcaacca

LU298      aggaggtctggcgccgccgcagcctcaggccactccccaggatcagcagatgggccctca    683
LU592      aggaggtctggcgcctccgcagcctcaggccactccccaggatcagcagatgggccctca    720
           aggaggtctggcgcc ccgcagcctcaggccactccccaggatcagcagatgggccctca

LU298      ccaccaccagatggctcagggacccccaggactgccagtgccgcctccccacccccaacgc    743
LU592      ccaccaccagatggctcaaggcctcccaggactgccggtccccccctccccacccccaacgc    780
           ccaccaccagatggctca gg c cccaggactgcc gt cc cctccccacccccaacgc

LU298      gcaacagcagcagcagcagcctcctc...ctcctcagcagcagcccccgtaccagcagca    800
LU592      gcagcagcagcagcagcagcaacagcagcccccctcagcagcagcctccctaccagcagca    840
           gca cagcagcagcagc  c  c   c cctcagcagcagcc cc taccagcagca

LU298      ggcctatccccagggccccggccccgtcgtgtccaacggcatgggtcctcagccgccgca    860
LU592      ggcctatcctcagggccctggccccgtcgtgtctaatggcatgggccctcagccgccgca    900
           ggcctatcc cagggccc ggccccgtcgtgtc aa ggcatggg cctcagccgccgca

LU298      gagcaccgcgtcgcccggcccaggtcgccgagccatcaaccgccccccaaacgccgtcgg    920
LU592      gagcaccgcgtcgcccggcccaggtcgccgagccatcaaccggcccccgaacgccgtcgg    960
           gagcaccgcgtcgcccggcccaggtcgccgagccatcaaccg ccccc aacgccgtcgg

LU298      gcctgcgacccccagatcaacacccccgtgccgtatcccaacaatgctcagtctccgca    980
LU592      ccctgcgacccctcagatcaacacccccgtgccgtatcccggcaatgctcagtctccgca   1020
            cctgcgacccc cagatcaacacccccgtgccgtatccc  caatgctcagtctccgca

LU298      agtgagccacccgacgcctgaccacgctcgcatgggtccccgcgcccctcccccctcccat   1040
LU592      agtgagccaccctacgccggatcacgctcgcatgggtccccgcgcccctcccccctcccat   1080
           agtgagccaccc acgcc ga cacgctcgcatgggtccccgcgcccctcccccctcccat

LU298      cagcaacgcgctgggcgacctcgaagttgatgccgttgcacctcacaataagaagacggg   1100
LU592      cagcaacgctctgggtgacctcgaagtcgacgccgtcgcgcctcataacaagaagacggg   1140
           cagcaacgc ctggg gacctcgaagt ga gccgt gc cctca aa aagaagacggg

LU298      caacgactggtatgccatcttcaacccgcaggtgcagcgcgtcctggatgtcgacctggt   1160
LU592      taacgactggtatgccattttcaacccgcaggtgcaacgcgttctggatgtcgacctggt   1200
            aacgactggtatgccat ttcaacccgcaggtgca cgcgt ctggatgtcgacctggt

LU298      ccactctctcacccacgagagcgtcgtttgctgtgttcgcttcagccacgacggcaagta   1220
LU592      ccactctctcacccacgagagcgttgtttgctgtgttcgctttagccacgacggcaagta   1260
           ccactctctcacccacgagagcgt gtttgctgtgttcgctt agccacgacggcaagta
```

```
LU298      tgtcgccaccggctgcaacaagtcggcccagattttcgatgtccagactggtgagaaggt   1280
LU592      cgtcgccactggctgcaacaagtcggcccagattttcgatgtccagactggtgagaaggt   1320
            gtcgccac ggctgcaacaagtcggcccagattttcgatgtccagactggtgagaaggt

LU298      gtgcgtcttggaggaccacagcgctaccgacatggctgccgatctctacattcgaagcgt   1340
LU592      gtgcgtcttggaagaccacagcgctaccgacatggctgccgatctatacattcgaagcgt   1380
           gtgcgtcttgga gaccacagcgctaccgacatggctgccgatct tacattcgaagcgt

LU298      ctgcttcagccctgacggccgctacctggctaccggtgccgaagacaagctgatccgagt   1400
LU592      ctgtttcagccctgacggccgctacctggccactggtgccgaagacaagctgatccgagt   1440
           ctg ttcagccctgacggccgctacctggc ac ggtgccgaagacaagctgatccgagt

LU298      aagtttgaccaatgatataatctaatggcggcacgctgttgtcactcat...........   1449
LU592      aagttaaaac....ataaaggataatggcggtagactggtgtcaccaacattactttctc   1496
           aagtt  a c    ata a   taatggcgg a  ctg tgtcac  a

LU298      ........tctccccatttcgcaagctgaccattggcgctacaggtgtgggatattgcta   1501
LU592      cctcatgccctccccgattcacaagctaaccatcggcgctacaggtgtgggatattgcta   1556
                    ctcccc  ttc caagct accat ggcgctacaggtgtgggatattgcta

LU298      cccgaaccatccgcaaccacttctcgggccacgaacaggacatttactcgctcgactttg   1561
LU592      cccgaaccatccgcaaccacttctcgggccacgaacaagacatttactcgctcgactttg   1616
           cccgaaccatccgcaaccacttctcgggccacgaaca gacatttactcgctcgactttg

LU298      cgcgcgacggccgcaccattgcctctggcagtggcgacagaacggtccgtctttgggata   1621
LU592      cacgcgacggccgtaccatcgcctctggcagtggcgatagaaccgtccgcctttgggata   1676
           c cgcgacggccg accat gcctctggcagtggcga agaac gtccg ctttgggata

LU298      ttgagcagggcaccaacaccctcaccctcaccatcgaggacggcgttacgactgttgcca   1681
LU592      ttgaacaaggcaccaacactctcaccctcaccattgaggacggtgtgacgactgttgcca   1736
           ttga ca ggcaccaacac ctcaccctcaccat gaggacgg gt acgactgttgcca

LU298      tttcccccgacacccagtttgttgcggccggctccctggacaagagcgttcgcgtctggg   1741
LU592      tttcccccgacactcagttcgttgcggccggctctctggacaagagcgttcgcgtctggg   1796
           tttcccccgacac cagtt gttgcggccggctc ctggacaagagcgttcgcgtctggg

LU298      acatcatgacaggctacctggtcgagcggttggaaggacccgatggacacaaggactctg   1801
LU592      acatcatgacaggctaccttgtcgagcggttggaaggacctgatggacacaaggactctg   1856
           acatcatgacaggctacct gtcgagcggttggaaggacc gatggacacaaggactctg

LU298      tctactccgttgctttctcgcccaacggcaaagacttggtcagcggcagtctggatcgaa   1861
LU592      tttattccgtcgctttctcgcccaacggcaaagacctggtcagcggcagtctggatcgaa   1916
           t ta tccgt gctttctcgcccaacggcaaagac tggtcagcggcagtctggatcgaa

LU298      cgattaagatgtgggagcttagctcgccccgcggcccgcagaactctggcgccaagggca   1921
LU592      cgatcaagatgtgggagcttagctcaccccgcggtccccagaactctggcgccaagggca   1976
           cgat aagatgtgggagcttagctc ccccgcgg cc cagaactctggcgccaagggca

LU298      agtgtgtcaagacattcgaaggccaccgagactttgtcctgtccgtcgccctgactccgg   1981
LU592      agtgtgtcaagacatttgagggccaccgagactttgtcctgtccgtcgccctgactccgg   2036
           agtgtgtcaagacatt ga ggccaccgagactttgtcctgtccgtcgccctgactccgg

LU298      acgccaactgggtcttgtccggatccaaggaccgtggcgtccagttctgggatccccgga   2041
LU592      acgctaactgggtcttgtcaggatccaaggaccgtggcgtccagttttgggacccccgga   2096
           acgc aactgggtcttgtc ggatccaaggaccgtggcgtccagtt tggga ccccgga

LU298      cggggactacccagctaatgcttcagggacataaaaactctgtcatctcagttgccccca   2101
LU592      caggaacaactcaactgatgcttcagggacacaagaactctgtcatctcagttgccccca   2156
           c gg ac ac ca ct atgcttcagggaca aa aactctgtcatctcagttgccccca

LU298      gcccacagggcggctactttgccaccggctcaggagacatgaaggctcgtatctggtcgt   2161
LU592      gcccacagggcggctacgttgccactggctcaggagacatgaaggcccgcatttggtcgt   2216
           gcccacagggcggctac ttgccac ggctcaggagacatgaaggc cg at tggtcgt

LU298      atcgtccatactag                                                 2175
LU592      atcgtccatactag                                                 2230
           atcgtccatactag
```

7.4.4 *Trichoderma* spp. *con-10*

7.4.4.1 *Trichoderma* spp. *con-10* Nucleotide Sequence Alignment

Partial alignment of the putative *con-10* nucleotide sequences isolated in this study from *T. atroviride* LU298 and *T. hamatum* LU592. The isolated region is situated approximately 33 bp 3' to the predicted translational start site to within 43 bp of the predicted stop codon. Consensus bases are in blue. Numbering is relative to this region only.

```
LU298      taaccggtacggtctcctacattacttttagcttccgaagctgaccatcaaaagccccaa     60
LU592      taaccggtatgacctttttggagcacttgtagatcttgcagctgaccatcaaaagacctaa     60
           taaccggta g  ct  t  a  actt tag t   g agctgaccatcaaaag cc aa

LU298      ggaggaggttcagaacattgccgccaagggtggaaaggctagtcacagcggcggctttgc    120
LU592      agaagaggtccaaaatattgcttccaagggcggacaggccagccacagcggcggcttcgc    120
            ga gaggt ca aa attgc  ccaaggg gga aggc ag cacagcggcggctt gc

LU298      ctccatggactctgataagcaggtcagtatctacatgttatgtgtaaattgtcgagatct    180
LU592      ttccatggatgctgataaacaggttagttattacagatttttccaggattga.aagattt    179
            tccatgga  ctgataa caggt agt   taca  tt t      attg   agat t

LU298      acgcgcattgacat..acatggcat...agcgtgaaatagcttctatgggaggaaaagcg    235
LU592      attcatattgactttaaaattgcattcaagcgcgaaatagcttctcaaggaggaaaagcc    239
           a  c  attgac t  a at gcat   agcg gaaatagcttct   ggaggaaaagc

LU298      tcgggaggatcttttgaaccgggcagt                                     262
LU592      tcgggaggatcttttgaacctggcagc                                     266
           tcgggaggatcttttgaacc ggcag
```

7.4.4.2 *Trichoderma* spp. *con-10* Amino Acid Alignment

Partial alignment of the Con-10 amino acid sequences from *T. atroviride* LU298 and *T. hamatum* LU592. The predicted amino acid sequences were derived using Wise2 (http://www.ebi.ac.uk/Wise2). The sequence begins approximately at protein position 12. Consensus amino acids are in red. Numbering is relative to this region only.

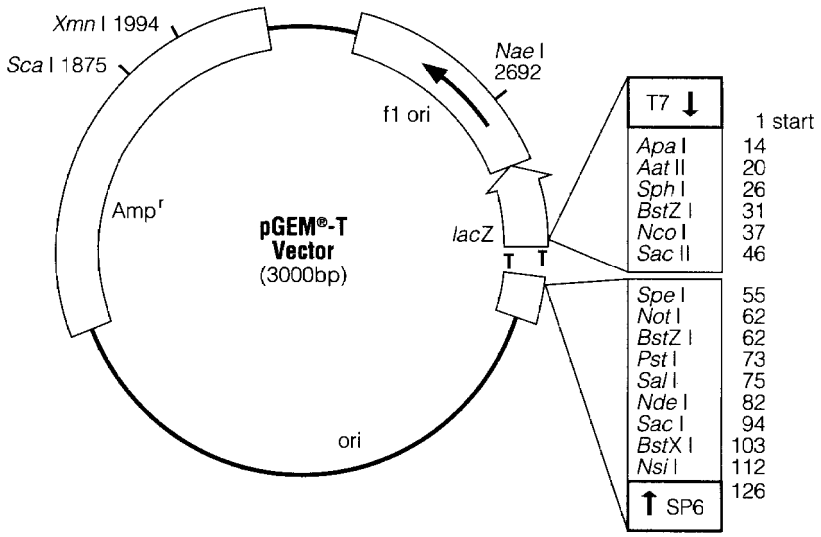
```
LU298      NRPKEEVQNIAAKGGKASHSGGFASMDSDKQREIASMGGKASGGSFEPGS              50
LU298      NRPKEEVQNIASKGGQASHSGGFASMDADKQREIASQGGKASGGSFEPGS              50
           NRPKEEVQNIA KGG ASHSGGFASMD DKQREIAS GGKASGGSFEPGS
```

7.5 Location of Putative DNA Binding Motifs within the Regulatory Regions of *rcoT* and Orthologues

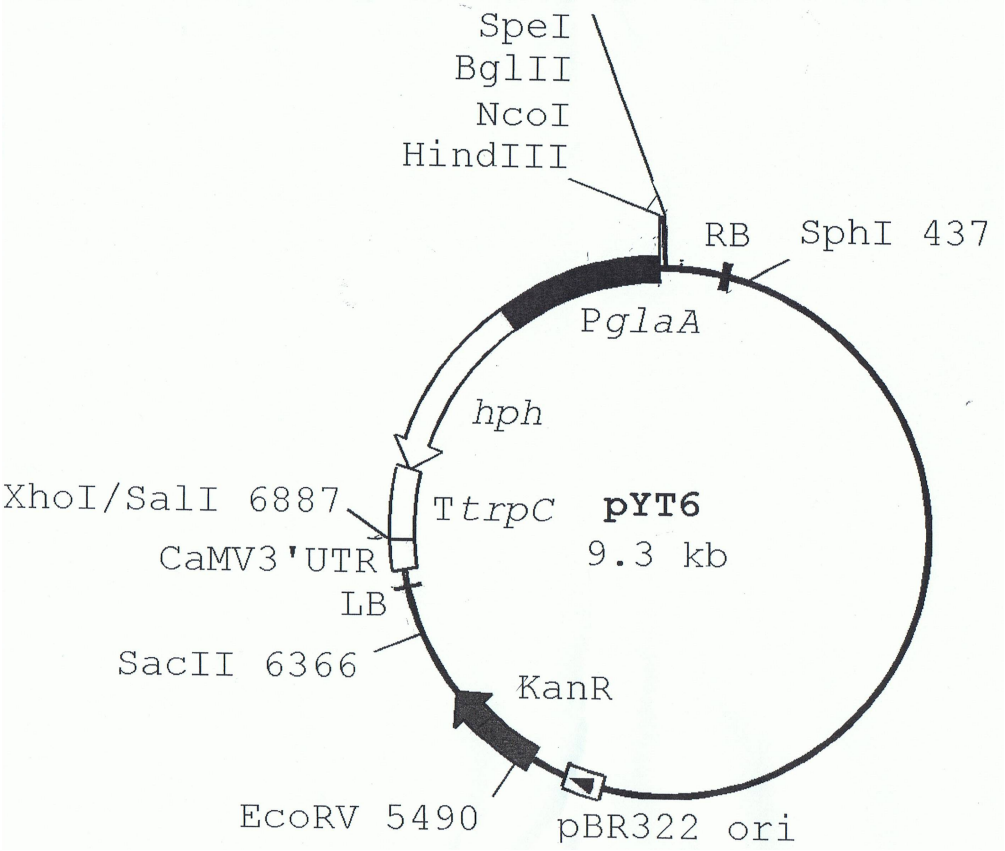
	caat	pacc	stress	gata	cre1	ct
T. reesei	-336	-912, -779	-1083, -1059, -958, -886, -723	-287	-1163, -1133, -973, -917, -827, -803, -698, -616, -594, -503, -260, -237, -82, -54	-866 to -908 -355 to -313
G. zeae	-1090	-672, -445, -172	-975, -845, -810, -607, -166	-1074, -70	-535, -514, -504	-421 to -369
M. grisea	-757	-798, -608, -594, -538, -44	-771, -375, -78	-247, -180	-896, -586, -395, -270	
N. crassa	-407, -357		-500, -372, -308	-	-1089, -1052, -905, -748, -628, -570, -483, -481, -199	-525 to -495
A. nidulans	-965, -762, -456, -312	-738, -210	-112, -78, -13	-842, -488, -419	-832, -825, -574, -325, -254, -75	-151 to -89

7.5 Plasmid Vectors

7.5.1 pGEM®-T



7.5.2 pYT6


SpeI
BglII
NcoI
HindIII
RB
SphI 437
PglaA
hph
XhoI/SalI 6887
TtrpC
pYT6
9.3 kb
CaMV3'UTR
LB
SacII 6366
KanR
EcoRV 5490
pBR322 ori